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Contents

Inhibition of Aldosterone Secretion and Modification of Electrolyte Excretion in Man by a Chemical Inhibitor of 11β-Hydroxylation. WILLIAM S. COPPAGE, JR., DONALD ISLAND, MAUREEN SMITH AND GRANT W. LIDDLE 2101

Studies on Isocitric and Lactic Dehydrogenases in Experimental Myocardial Infarction. PAUL E. STRANDJORD, KENNETH E. THOMAS AND LAURENS P. WHITE 2111

Studies of Colon Contractility in Dogs. I. Description of Method and Effect of Parasympathomimetic Agents and Serotonin. MARVIN H. SLEISENGER, DAVID H. LAW, FREDERICK W. SMITH, JAMES H. PERT AND CHARLES M. LEWIS 2119

The Effect of Insulin on Nonesterified Fatty Acid Release from the Human Leg. E. H. ESTES, JR., M. D. BOGDONOFF, S. J. FRIEDBERG, W. R. HARLAN, JR. AND D. L. TROUT 2131

Electrophoretic Studies of the Vitamin B12-Binding Protein of Normal and Chronic Myelogenous Leukemia Serum. AARON MILLER AND JOHN F. SULLIVAN 2135

The Idiopathic High Cardiac Output State. RICHARD GORLIN, NORMAN BRACHFELD, JOHN D. TURNER, JOSEPH V. MESSER AND EDUARDO SALAZAR 2144

A Study in Man of Cerebral Blood Flow and Cerebral Glucose, Lactate and Pyruvate Metabolism Before and After Eating. GEORGE G. ROWE, GEORGE M. MAXWELL, CESAR A. CASTILLO, D. J. FREEMAN AND CHARLES W. CRUMPTON 2154

Physicochemical and Immunologic Studies of Gamma Globulins of Normal Human Urine. E. C. FRANKLIN 2159

Static Volume-Pressure Relations of Excised Lungs of Infants with Hyaline Membrane Disease, Newborn and Stillborn Infants. IRWIN GRIBETZ, N. R. FRANK AND MARY ELLEN AVERY 2168

Interrelations of Plasma Potassium Concentration, Plasma Sodium Concentration, Arterial pH and Total Exchangeable Potassium. J. LEIBMAN AND I. S. EDELMAN 2176

Hepatic Metabolism of Phenylalanine during Development. FRANCIS T. KENNEY AND NORMAN KRETCHMER 2189

Renal Reabsorption of Bicarbonate During Acute Respiratory Alkalosis. WILLIAM B. SCHWARTZ, GUY LEMIEUX AND ADRIEN FALBRIARD 2197

Studies Concerning Functional Differences Between Liver Regions Supplied by the Hepatic Artery and by the Portal Vein. RALPH W. BRAUER, OTTO S. SCHILL AND JOHN S. KREBS 2211

The Relation of Sulfur Metabolism to Acid-Base Balance and Electrolyte Excretion: The Effects of DL-Methionine in Normal Man. JACOB LEMANN, JR. AND ARNOLD S. RELMAN 2215

Pulmonary Transcapillary Exchange of Na24 and P32-Labeled Phosphate in Pulmonary Emphysema. M. A. ROTHSCHILD, A. L. DAVIS, M. ORATZ AND S. S. SCHREIBER 2224

Metabolic Patterns During Physiologic Sleep: I. Blood Glucose Regulation During Sleep in Normal and Diabetic Subjects. EUGENE D. ROBIN, DAVID M. TRAVIS, DESMOND G. JULIAN AND BURIS R. BOSHELL 2229

A Study of Subjects with Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency. II. Investigation of Leukocyte Enzymes. BRACHA RAMOT, SHARON FISHER, ARYEH SZEINBERG, AVINOAM ADAM, CHAIM SHEBA AND DORA GAFNI 2234

Cortisol Metabolism in Man: Observations of Pathways, Pool Sizes of Metabolites and Rates of Formation of Metabolites. NORMAN I. GOLD, LOUIS L. SMITH AND FRANCIS D. MOORE 2238

Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency: Evidence of Differences Between Negroes and Caucasians with Respect to this Genetically Determined Trait. PAUL A. MARKS AND RUTH T. GROSS 2253

Author Index to Volume XXXVIII 2263

Subject Index to Volume XXXVIII 2284

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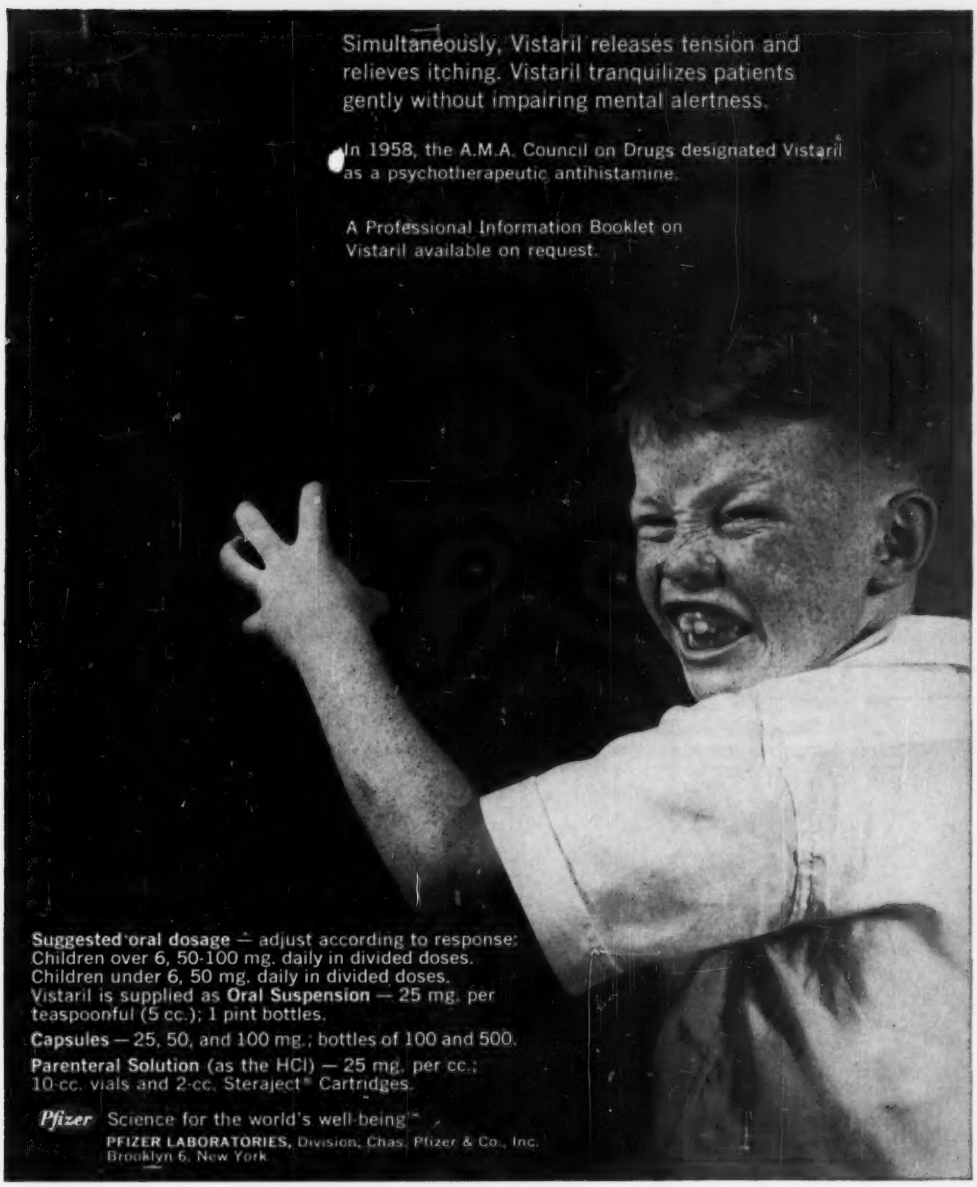

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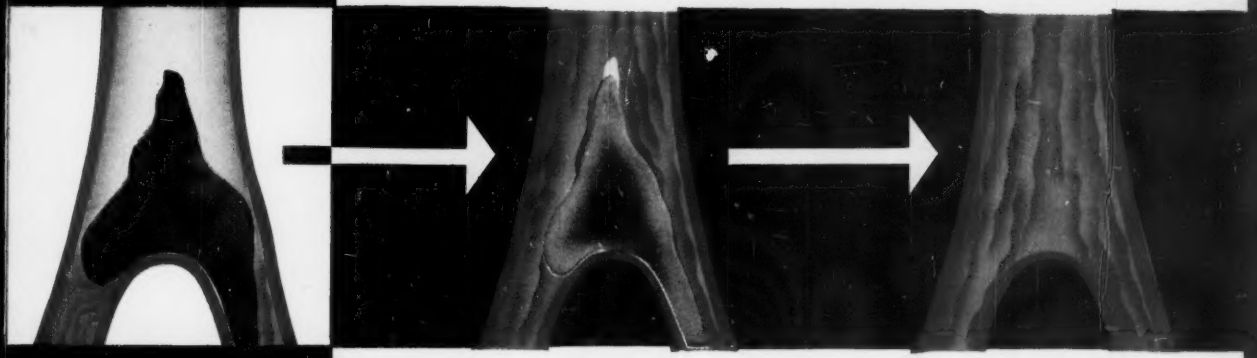

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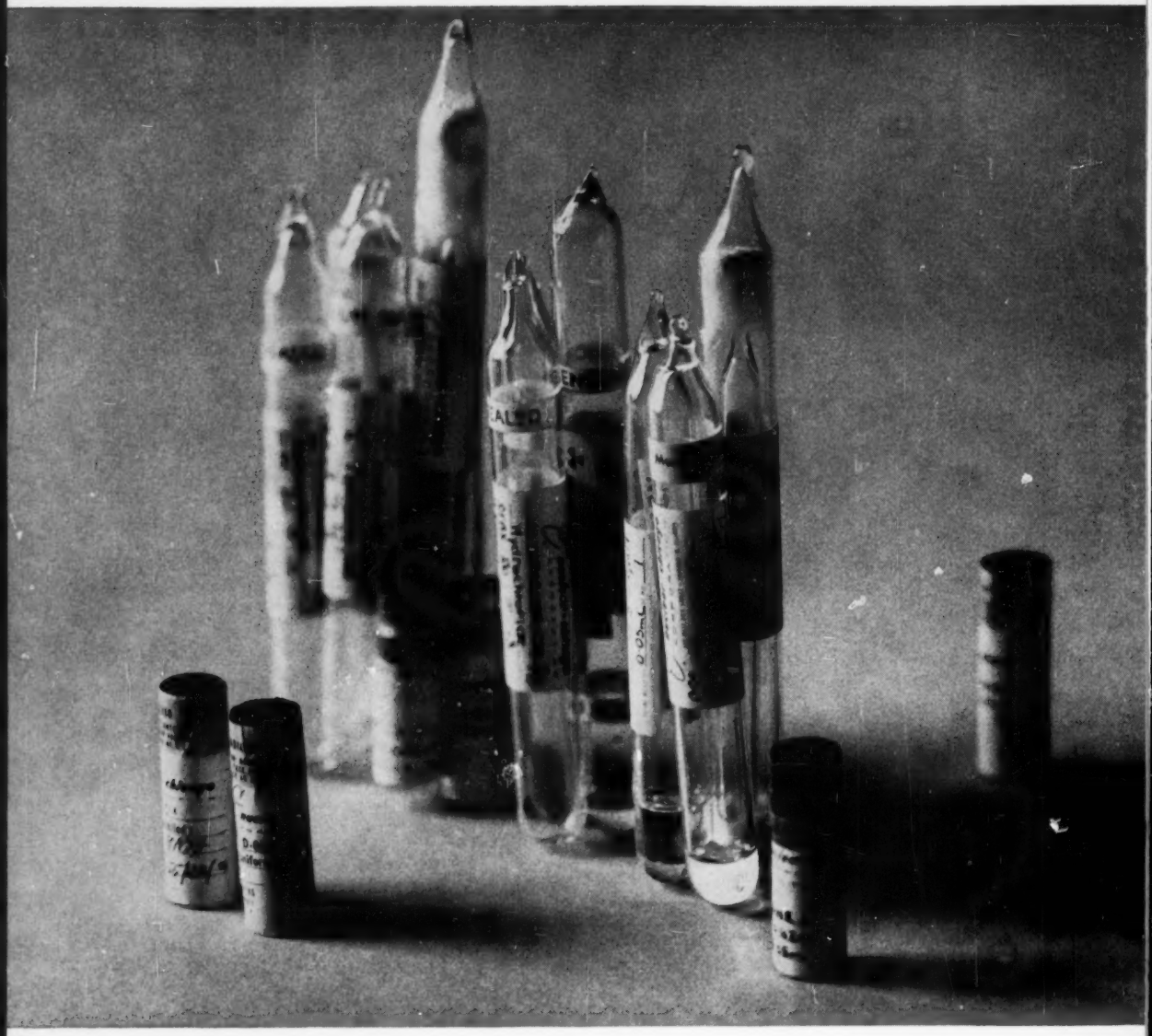

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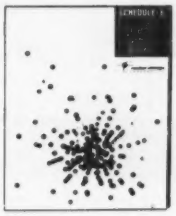

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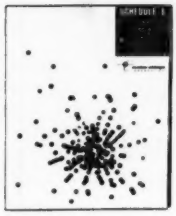

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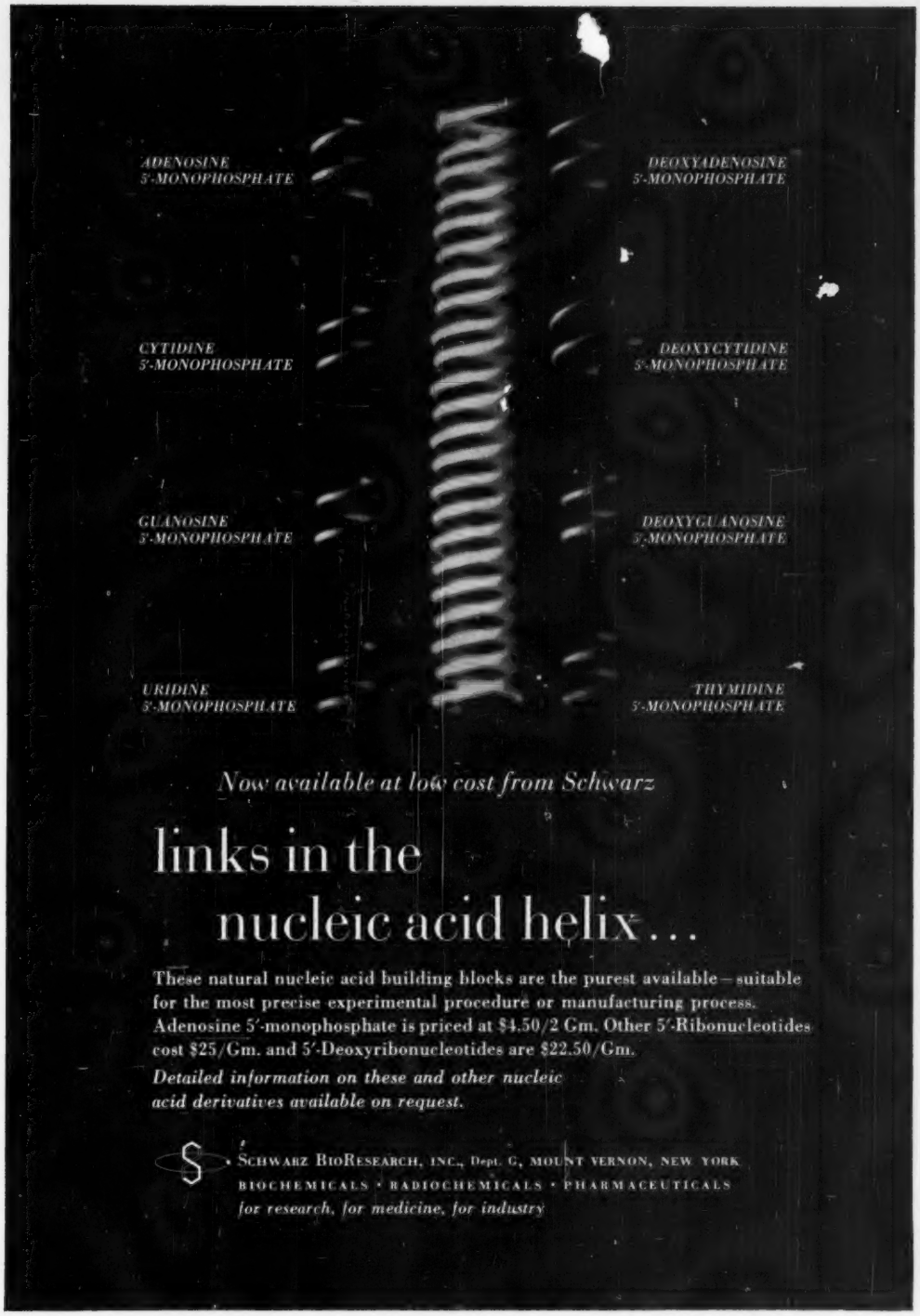

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INHIBITION OF ALDOSTERONE SECRETION AND MODIFICATION OF ELECTROLYTE EXCRETION IN MAN BY A CHEMICAL INHIBITOR OF 11β-HYDROXYLATION * †

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In 1958 it was shown by Chart and associates (1) that 2-methyl-1,2-bis-(3-pyridyl)-1-propanone (SU-4885) (Figure 1) when administered to dogs resulted in a striking, acute decrease in adrenocortical secretion of cortisol. Subsequent studies in the dog (2) and in man (3) indicated that SU-4885 inhibited the 11β-hydroxylation of corticosteroids so that 11-desoxycortisol (compound S) tended to replace cortisol and 11-desoxycorticosterone tended to replace corticosterone in the adrenal effluent. The ring-A saturated (tetrahydro-) metabolites of compound S and desoxycorticosterone (DOC) are not ordinarily present in measurable quantities in the urine, but during treatment with SU-4885 these steroids have been identified as major urinary steroids (3).

In man, cortisol is the adrenal hormone of principal importance in restraining adrenocorticotropic hormone (ACTH) secretion by the pituitary. By inducing a decrease in cortisol secretion, therefore, SU-4885 indirectly induces a measurable increase in ACTH secretion (4). Under the influence of high levels of ACTH the SU-4885-inhibited adrenal gland secretes large quantities of 11-desoxycorticosteroids (Figure 2).

The steroid patterns produced by SU-4885 are similar to those observed by Eberlein and Bongiovanni (5) in the hypertensive form of congenital adrenal hyperplasia, in which disease it is thought that 11β-hydroxylation is defective, leading to a relative or absolute deficiency of cortisol secretion, a compensatory increase in ACTH secretion, and greatly excessive secretion of compound S, DOC and other 11-desoxysteroids.

Since aldosterone is hydroxylated in the 11β position, one might anticipate that its secretion would be inhibited by SU-4885 (Figure 3). A decrease in secretion of this important mineralocorticoid might be expected to lead to an increase in urinary sodium. The present report is concerned with a description of the effects of SU-4885 upon mineralocorticoid and electrolyte metabolism in man.

MATERIALS AND METHODS

Six normal young adults and nine hospitalized patients were studied while receiving diets which were constant from day to day. Complete 24 hour urine specimens, collected on ice or with thymol preservative, were analyzed within several hours after collection or were frozen until future analysis. SU-4885 was administered orally. Whenever steroids were employed they too were administered orally. Urinary aldosterone was measured by a modification of the method of Kliman and Peterson (6). Urinary pregnane-3,18,21-triol-11,20-dione was determined by a modification of the method of Ulick, Laragh and Lieberman (7). 17,21-Dihydroxy-20-ketosteroids (17-hydroxycorticoids) were determined by the method of Silber and Porter (8). 17-Ketosteroids were measured utilizing a color correction equation modified from Allen (9) applied to a methylene chloride extract of the Zimmermann chromogen developed by the procedure of Callow, Callow and Emmens (10) on urine ex-

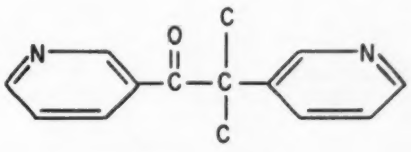

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FIG. 1. CHEMICAL STRUCTURE OF 2-METHYL-1 2-BIS-(3-PYRIDYL)-1-PROPANONE (SU-4885)

* Presented in part at the Annual Meeting of the Southern Society for Clinical Research, New Orleans, La., January 24, 1959.

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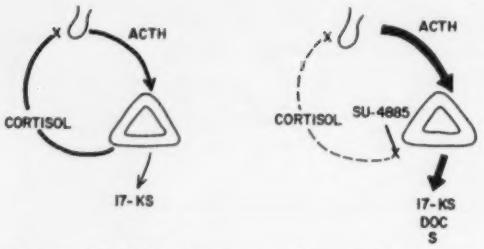

FIG. 2. SU-4885 INHIBITION OF CORTISOL RESULTS IN "COMPENSATORY" INCREASE IN ACTH SECRETION

ACTH in the presence of 11β-hydroxylase inhibition stimulates secretion of 11-desoxycorticosteroids such as 11-desoxycortisol (compound S) and DOC.

tracts prepared by the method of Drekter and associates (11). Steroid metabolites in the urine (tetrahydrocortisol, tetrahydrocortisone, tetrahydrodesoxycorticosterone and tetrahydro-compound S) were determined by methods previously described (3). Urinary chloride was determined by the potentiometric method of Sanderson (12). Sodium and potassium were analyzed by flame photometry. Urinary creatinine was determined by the method of Hawk, Oser and Summerson (13).

RESULTS

Regardless of the experimental conditions, SU-4885 consistently caused decreases in urinary aldosterone levels.

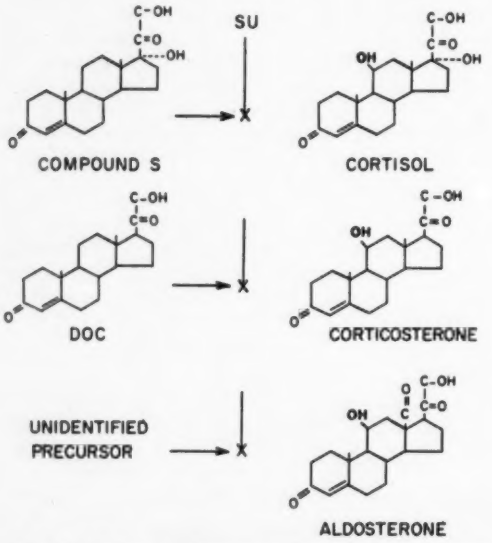

FIG. 3. CORTISOL, CORTICOSTERONE AND ALDOSTERONE ARE ALL HYDROXYLATED IN THE 11β-POSITION AND ARE THEREFORE SUSCEPTIBLE TO SU-4885 INHIBITION

In individuals with intact pituitary-adrenal function the administration of SU-4885 consistently resulted in the appearance of DOC in the blood and its metabolite tetrahydrodesoxycorticosterone in the urine. That this appearance of DOC was dependent upon increased secretion of ACTH has been shown previously (3) and was confirmed in this study by the fact that SU-4885 administration failed to result in the appearance of DOC in blood or urine when ACTH secretion was suppressed by prednisone or Dexamethasone®.

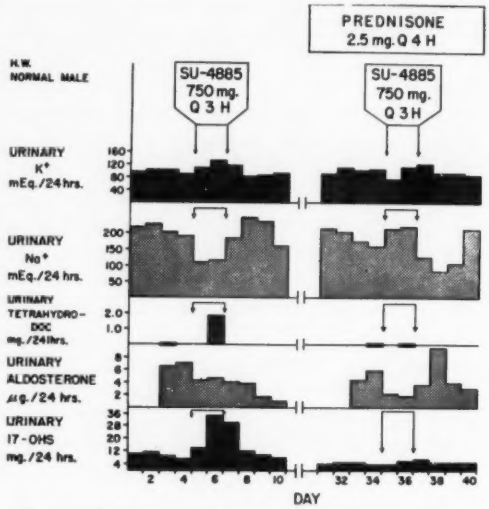

FIG. 4. IN NORMAL INDIVIDUAL ON LIBERAL SODIUM INTAKE SU-4885 ALONE CAUSED DOC SECRETION WITH SODIUM RETENTION AND POTASSIUM LOSS

When prednisone was administered before and during SU-4885, no DOC was secreted and aldosterone inhibition was accompanied by sodium loss and potassium retention.

Effects of SU-4885 in normal subjects on a liberal sodium intake

Two normal individuals maintained on a constant high sodium intake received SU-4885. One of these subjects had a daily sodium intake of 200 mEq. and received SU-4885 750 mg. every three hours for two days. The second subject had a daily sodium intake of 100 mEq. and was given SU-4885 500 mg. every four hours for three days. Both studies are tabulated in Table I. Figure 4 illustrates the results of the first study.

When Subject H. W. received SU-4885 as his only treatment, tetrahydro-DOC, which had been

TABLE I

Effects of SU-4885 on urinary electrolytes and steroids in normal subjects on liberal sodium intake*

Day	Treatment	Creatinine	Chloride	Potassium	Sodium	17-Hydroxycorticosteroids	17-Ketosteroids	Aldosterone	Tetrahydrocortisone plus tetrahydrocortisol	Tetrahydrocompound S	Tetrahydrodesoxycorticosterone
No.		*mg./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mg./day*	*mg./day*	*µg./day*	*mg./day*	*mg./day*	*mg./day*
H.W., 27, normal man; 200 mEq. sodium diet											
1	0	1,860	204	97	222	10	12				
2	0	1,790	206	102	230	11	7				
3	0	2,100	177	99	205	9	13	7	4.6	0	0
4	0	1,720	166	89	193	7	15	7			
5	SU-4885, 750 mg. every 3 hrs.	2,070	118	110	112	14	16	4			
6	SU-4885, 750 mg. every 3 hrs.	2,080	103	136	117	35	29	4	4.0	13	1.8
7	0	1,960	168	118	187	30	28	4			
8	0	1,990	208	82	252	12	17	4			
9	0	2,160	215	86	240	10	11	2			
10	0	2,050	163	92	161	8	8	1			
31	Prednisone 2.5 mg. every 4 hrs.	1,990	184	89	218	4	10				
32	Prednisone 2.5 mg. every 4 hrs.	2,100	190	110	207	5	7				
33	Prednisone 2.5 mg. every 4 hrs.	2,100	150	104	179	5	5	4			
34	Prednisone 2.5 mg. every 4 hrs.	1,960	131	108	160	4	5	6		0	0
35	Prednisone 2.5 mg. every 4 hrs. and	2,260	179	76	216	4	5	2			
36	SU-4885, 750 mg. every 3 hrs.	2,060	210	116	222	6	8	2		0	0
37	Prednisone 2.5 mg. every 4 hrs.	2,040	84	124	129	7	14	4			
38	Prednisone 2.5 mg. every 4 hrs.	2,000	82	94	86	5	7	10			
39	Prednisone 2.5 mg. every 4 hrs.	2,180	90	94	108	5	5	4			
40	Prednisone 2.5 mg. every 4 hrs.	1,950	191	90	218	5	5	3			
J.J., 25, normal man; 100 mEq. sodium diet											
1	0	2,480	186	125	103	14	14				
2	0	2,350	220	132	101	13	14				
3	0	2,380	188	116	96	13	14	22			
4	0	2,430	190	125	85	13	14	17	5.4	0	0
5	SU-4885, 500 mg. every 4 hrs.	2,260	181	109	107	17	9	22			
6	SU-4885, 500 mg. every 4 hrs.	2,340	178	108	79	17	12	4			
7	SU-4885, 500 mg. every 4 hrs.	2,460	184	135	91	30	19	4	5.7	8.2	1.3
8	0	2,560	180	114	101	14	14	21			
9	0	2,220	194	114	109	12	10	18			
10	0	2,400	203	118	103	12	10				

* SU-4885 = 2-methyl-1, 2-bis-(3-pyridiyl) 1-propanone.

too low to measure during control periods, rose to easily appreciable levels. Simultaneously, large quantities of tetrahydro-S appeared in the urine, accounting for a large increase in total 17-hydroxycorticoid excretion. Associated with the increase in DOC and compound S secretion there was a significant decrease in urinary sodium and an increase in urinary potassium. Urinary aldosterone fell with SU-4885 administration. Presumably the sodium retention and potassium loss were consequences of the increase in DOC secretion.

A somewhat different response was observed when treatment with SU-4885 was repeated while H.W. was receiving prednisone as a suppressor of ACTH. Under these conditions SU-4885 did not induce the appearance of measurable quantities of tetrahydro-DOC or tetrahydro-S in the urine. Once again urinary aldosterone fell with SU-4885 administration. Sodium and chloride excretion increased and potassium excretion decreased, presumably as consequences of decreased aldosterone secretion.

TABLE II

Effects of SU-4885 and prednisone on urinary electrolytes and steroids in two normal subjects on constant 9 mEq. sodium diets*

Day	Treatment	Creatinine	Chloride	Potassium	Sodium	17-Hydroxycorticosteroids	17-Ketosteroids	Aldosterone	Pregnan-3, 18, 21-triol-11, 20-dione	Tetrahydrocortisone plus tetrahydrocortisol	Tetrahydrocompound S	Tetrahydrodesoxycorticosterone
No.		*mg./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mg./day*	*mg./day*	*µg./day*	*µg./day*	*mg./day*	*mg./day*	*mg./day*
H.W., 27, normal man												
1	Prednisone 2.5 mg. every 8 hrs.	1,560	3	50	2	4	8	15	50			
2	Prednisone	1,330	3	48	1	4	9	14	43			
3	Prednisone	1,460	4	51	1	4	8	11	43			
4	Prednisone	1,420	4	47	2	4	7	18	49	0.9	0	0
5	Prednisone 2.5 mg. every 8 hrs. and	1,470	5	39	10	5	8	3	10			
6		1,730	6	81	7	12	11	6	11			
7	SU-4885, 500 mg. every 4 hrs.	1,460	7	83	15	12	13	6	0	1.8	5.7	0.9
8	Prednisone 2.5 mg. every 8 hrs.	1,430	8	67	7	8	8	8	37			
9	Prednisone	1,880	6	47	4	4	8	15	45			
10	Prednisone	1,260	5	46	3	4	7	15	42			
11	Prednisone	1,480	6	60	3	4	7	15	50			
12	Prednisone	1,500	8	71	4	5	5	14				
13	Prednisone	1,480	6	56	4	3	5	9				
14	Prednisone 2.5 mg. every 4 hrs.	1,415	5	51	2	3	5	9				
15	Prednisone	1,440	5	50	3	3	5	15				
16	Prednisone	1,430	5	52	3	3	7	15				
17	Prednisone	1,375	5	51	7	2	5	13				
18	Prednisone	1,690	5	57	5	2	9	13	30	0.13	0	0
19	Prednisone 2.5 mg. and	1,310	6	33	32	4	6	3	4			
20	SU-4885, 500 mg.	1,690	11	42	38	4	6	8	4			
21	every 4 hrs.	1,495	11	47	31	4	6	3	15	0.17	0	0
22	Prednisone 2.5 mg. every 4 hrs.	1,770	17	77	17	3	5	16	26			
23	Prednisone	1,770	7	69	19	3	6	20	38			
24	Prednisone	1,715	8	60	3	4	4	11				
25	Prednisone	1,575	7	51	2	2	4	20				
J.J., 25, normal man												
1	Prednisone 2.5 mg. every 4 hrs.	1,810	13	112	7	7	11					
2	Prednisone	1,716	8	104	2	7	5	72				
3	Prednisone	1,760	11	137	3	8	9	47				
4	Prednisone	1,610	8	112	1	9	8	79				
5	Prednisone	2,120	8	121	2	8	7	62				
6	Prednisone	2,680	6	125	1	7	9	59				
7	Prednisone	2,440	6	136	2	7	7	39				
8	Prednisone	1,760		107	2	5	8	62				
9	Prednisone 2.5 mg. every 4 hrs. and	2,230	14	111	11	13	11	23				
10		1,950	9	91	5	10	11	6				
11	SU-4885, 500 mg. every 4 hrs.	1,910	12	121	9	9	14	11				
12	Prednisone 2.5 mg. every 4 hrs.	1,915	12	73	1	8	10	6				
13	Prednisone	1,610	9	64	1	6	10	31				
14	Prednisone	1,670	6	100	0.4	6	10	70				
15	Prednisone	1,600	6	120	0.5	5	8	56				
16	Prednisone	1,635	4	106	0.4	5	6	85				
22	Prednisone 5 mg. every 4 hrs.	1,920	16	103	1	6	8					
23	Prednisone	1,680	10	127	1	6	9	43				
24	Prednisone	1,880	8	93	0.5	5	8	41				
25	Prednisone	1,905	12	136	1	6	7	58		0.27	0	0
26	Prednisone 5 mg. every 4 hrs. and	1,850	9	78	3	6	7	25				
27		1,950	21	103	25	8	7	8				
28	SU 4885, 500 mg. every 4 hrs.	1,630	26	99	15	7	8	13		0.41	0	0
29	Prednisone 5 mg. every 4 hrs.	1,720	16	110	3	6	7	19				
30	Prednisone	1,670	11	106	0.7	6	7	38				
31	Prednisone											
32	Prednisone	1,800	13	124	0.5	8	7	68				

* See footnote Table I.

When Subject J. J. received SU-4885 alone, tetrahydro-DOC and tetrahydro-S appeared in the urine in large quantities and once again there was a definite decrease in urinary aldosterone. Little change was noted in urinary sodium, chloride or potassium levels. Presumably in this particular study the rise in DOC secretion offset the fall in aldosterone with respect to effects on electrolyte excretion.

Effects of SU-4885 in normal subjects with high aldosterone levels secondary to low sodium intake

Restriction of dietary sodium in the normal individual has been shown to result in a decrease in urinary sodium and a rise in urinary aldosterone, which in our experience has been maintained for as long as sodium is rigidly withheld. One normal young woman taking a constant diet containing 9 mEq. of sodium per day was treated with SU-4885 in dosage of 500 mg. every four hours for two days. No ACTH suppressor was employed. During treatment with SU-4885 urinary tetrahydrodesoxycorticosterone rose from unmeasurable levels to 1.1 mg. per 24 hours. Urinary aldosterone fell from 21 μg. prior to treatment to 6 μg. per 24 hours on the first day and then rose again to 20 μg. on the second day of treatment. No significant changes in urinary sodium or potassium were noted during SU-4885 administration.

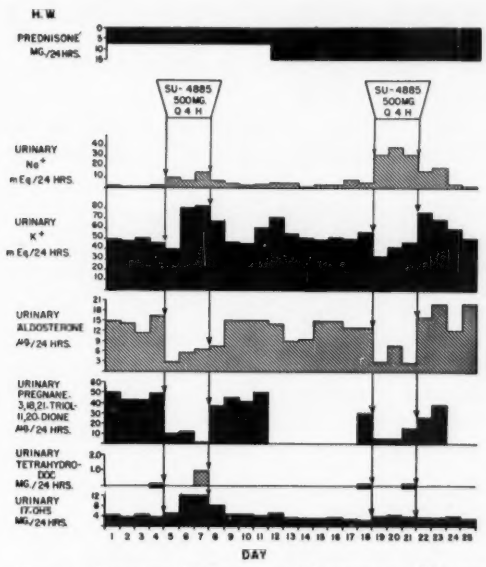

FIG. 5. URINARY STEROID AND ELECTROLYTE VALUES OF A NORMAL MAN MAINTAINED ON A CONSTANT LOW SODIUM DIET

During the first portion of the study prednisone in dosage of 2.5 mg. every eight hours failed to suppress completely the secretion of DOC in response to SU-4885. Although aldosterone levels fell, sodium diuresis was trivial and potassium excretion increased. During the second portion of the study prednisone in dosage of 2.5 mg. every four hours prevented secretion of DOC, so that as aldosterone levels fell there occurred a marked increase in urinary sodium accompanied by a decrease in urinary potassium.

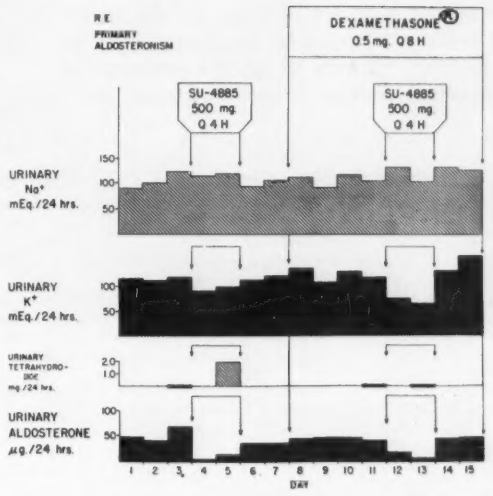

FIG. 6. IN THIS PATIENT WITH PRIMARY ALDOSTERONISM SU-4885 INHIBITION OF ALDOSTERONE WAS ASSOCIATED WITH POTASSIUM RETENTION

The effect was more marked when DOC secretion was precluded by Dexamethasone®.

Two other normal subjects on low sodium diet were studied while receiving prednisone as a suppressor of ACTH. The results are tabulated in Table II. Figure 5 illustrates one such study, which can best be considered in two parts. In the first half of the study the subject received only 7.5 mg. prednisone per day. That this was insufficient to suppress ACTH secretion completely is evidenced by the marked increase in urinary levels of tetrahydrodesoxycorticosterone and tetrahydro-compound S which occurred during treatment with SU-4885. Despite the fact that urinary aldosterone decreased from 18 μg. per 24 hours to

less than 3 μg. per 24 hours and the urinary metabolite of aldosterone, pregnane-3,18,21-triol-11, 20-dione, fell from 49 μg. to less than 10 μg. per 24 hours, sodium diuresis was trivial and potassium excretion increased during treatment with SU-4885.

During the second half of the study the dose of prednisone was increased to 15 mg. per day.

TABLE III

Effects of SU-4885, with and without ACTH-suppressing steroids, in patients with secondary hyperaldosteronism*

Day	Treatment	Volume	Potassium	Sodium	17-Hydroxycorticosteroids	17-Ketosteroids
No.		*ml./day*	*mEq./day*	*mEq./day*	*mg./day*	*mg./day*
L.T., 50, Laennec's cirrhosis, ascites; 22 mEq. sodium diet						
1	0	3,350	97	5.7	7	1
2	0	2,940	75	4.4	6	2
3	0	2,960	80	0.5	7	4
4	SU-4885, 750 mg. every 4 hrs.	2,100	47	1.3	18	3
5		2,200	73	1.5	36	8
6	0	2,850	80	0.6	11	7
7	0	3,195	78	0.5	7	4
J.W., 75, arteriosclerotic heart disease, edema; 22 mEq. sodium diet						
1	0	1,160	42	29	6	4
2	0	1,400	28	31	4	2
3	0	2,225	42	28	6	3
4	SU-4885, 500 mg. every 4 hrs.	1,750	47	9	10	4
5		2,160	57	7	20	4
6	0	1,950	77	20	28	9
7	0	750	38	9		4
J.M., 40, nephrosis, edema; 22 mEq. sodium diet						
1	Prednisone	2,000	72	12		
2	Prednisone	1,500	40	9		
3	Prednisone, 5 mg. every 8 hrs.	1,710	30	9		
4	Prednisone	1,770	34	11		
5	Prednisone, 5 mg every	1,310	28	10		
6	and	1,920	34	20		
7	SU-4885, 500 mg. eve[illegible] 4 hrs.	2,110	33	32		
8	Prednisone	1,760	34	28		
9	Prednisone	1,550	37	18		
10	Prednisone, 5 mg. every 8 hrs.	1,670	27	16		
11	Prednisone	1,530	44	11		
12	Prednisone	1,650	54	12		
L.M., 36, postnecrotic cirrhosis, ascites; 22 mEq. sodium diet						
1	0	1,180	64	0.2	2	1
2	0	1,260	68	0.2	2	2
3	0	1,520	65	0.2	3	1
4	Dexamethasone^R	1,480	94	0.3	3	1
5	Dexamethasone^R, 0.5 mg. every 8 hrs.	1,460	66	0.3	1	1
6	Dexamethasone^R	1,290	69	0.3	2	1
7	Dexamethasone^R 0.5 mg. every 8 hrs.	1,350	61	5.4	2	1
8	and	1,510	73	7.6	2	2
9	SU-4885, 750 mg. every 3 hrs.	1,385	60	7.5	3	1
10	Dexamethasone^R	1,085	55	1.6	1	1
11	Dexamethasone^R, 0.5 mg. every 8 hrs.	1,345	60	0.7	2	1
12	Dexamethasone^R	1,100	35	0.5	2	1
13	Dexamethasone^R	1,180	64	0.7	3	3

* See footnote, Table I.

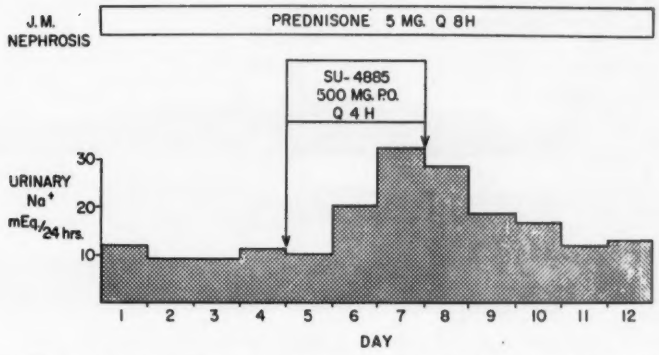

FIG. 7. IN THIS PATIENT WITH NEPHROSIS AND SECONDARY HYPERALDOSTERONISM, SU-4885 INHIBITION OF ALDOSTERONE RESULTED IN SODIUM DIURESIS WHEN DOC SECRETION WAS PREVENTED BY PREDNISONE

This time no tetrahydrodesoxycorticosterone was measurable in the urine either before or during SU-4885 treatment. Once more SU-4885 induced a striking fall in urinary aldosterone and pregnane-3,18,21-triol-11,20-dione; this time the fall in aldosterone was accompanied by an unequivocal increase in urinary sodium and decrease in urinary potassium.

Effects of SU-4885 in a patient with primary hyperaldosteronism

The effect of SU-4885 was studied in one patient with primary hyperaldosteronism, proven at operation to be due to a benign adrenocortical adenoma (Figure 6). The patient was a 32 year old woman with hypertension, cardiac arrhythmias, and hypokalemia despite potassium supplements. On a constant diet containing 100 mEq. sodium and 128 mEq. potassium, here urinary aldosterone averaged 49 μg. per 24 hours. In the absence of any ACTH suppressors, SU-4885 in dosage of 500 mg. every four hours for two days resulted in a striking fall in aldosterone to 4 μg. per day and a rise in tetrahydrodesoxycorticosterone to 1.9 mg. per day. Urinary potassium decreased slightly.

In a subsequent portion of the study the patient was given Dexamethasone® 0.5 mg. every eight hours as a suppressor of ACTH. Again a marked fall in urinary aldosterone was observed during treatment with SU-4885 but this time a measurable quantity of tetrahydrodesoxycorticosterone failed to appear in the urine. Urinary potassium fell sharply during treatment with SU-4885 and rose again following withdrawal of SU-4885. Despite the impressive effect of SU-4885 on urinary aldosterone and potassium, no appreciable change in urinary sodium occurred.

Effects of SU-4885 in patients with secondary hyperaldosteronism

Four patients with secondary hyperaldosteronism and edema have been studied (Table III). In Patient L.T., a man with Laennec's cirrhosis, ascites, and marked sodium retention, the administration of SU-4885 without any ACTH suppressor caused urinary potassium to fall from 80 mEq. per day to 47 mEq. per day, but urinary sodium did not change significantly. In Patient J.W., a man with arteriosclerotic heart disease and edema, sodium retention occurred in response to SU-4885 administered in the absence of any suppressors of ACTH. Two edematous patients, J.M. and L.M., were receiving ACTH-suppressing steroids when SU-4885 was administered. In both cases treatment with SU-4885 resulted in a significant increase in sodium excretion (Figure 7). In patients with secondary hyperaldosteronism and near-maximal tubular reabsorption of sodium the SU-4885-induced increase in sodium excretion has sometimes been as much as 20-fold over baseline excretion, but even this has not constituted enough of a diuresis to be worthwhile from the standpoint of clinical therapy.

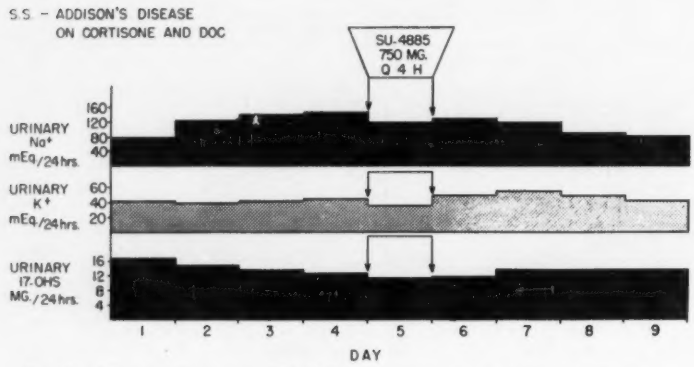

FIG. 8. LACK OF EFFECT OF SU-4885 IN PATIENT WITH ADDISON'S DISEASE

This patient was maintained on a constant high sodium diet, cortisone acetate 50 mg. (intramuscular) daily and DOC-trimethylacetate 50 mg. (intramuscular) every four weeks.

SU-4885 administration in Addison's disease

If it is assumed that the effects of SU-4885 on sodium and potassium excretion are the manifestations of altered mineralocorticoid metabolism due to inhibition of adrenocortical 11β-hydroxylase, the presence of the adrenal cortex is a requisite for its action. To exclude the possibility that SU-4885 might have some extra-adrenal electrolyte-regulating function, it was given to a bilaterally adrenalectomized patient maintained on salt, cortisone and DOC-trimethylacetate (Figure 8). There was no significant change in urinary sodium, potassium, 17-hydroxycorticoids or 17-ketosteroids.

DISCUSSION

SU-4885 is a potent inhibitor of 11β-hydroxylation of adrenal steroids. By inhibiting 11β-hydroxylation it interferes with the secretion of three major adrenal steroids—cortisol, corticosterone and aldosterone. The level of circulating cortisol controls ACTH release. Therefore a "compensatory" increase in ACTH secretion occurs as a consequence of the SU-4885-induced decrease in cortisol secretion. In the dosages employed in this study SU-4885 does not completely block 11β-hydroxylation, so that under the influence of high levels of ACTH the secretion of cortisol gradually (within a day or so) returns to normal. At the same time, however, the high levels of ACTH acting upon the SU-4885-inhibited adrenals bring about the secretion of compound S and desoxycorticosterone at rates far beyond the normal range. Compound S has neither significant glucocorticoid nor mineralocorticoid activity (14), but DOC is a potent mineralocorticoid. SU-4885 treatment of individuals with normal pituitary function results, then, in inhibition of one mineralocorticoid, aldosterone, and enhanced secretion of another, desoxycorticosterone. When DOC secretion predominates, sodium retention and potassium loss occur. When aldosterone *inhibition* predominates, sodium loss and potassium retention occur. The net effect of SU-4885 on electrolytes represents the algebraic sum of its DOC-promoting and aldosterone-inhibiting actions.

The inhibition of aldosterone by SU-4885 is a primary effect, *i.e.*, a consequence of direct action of the compound on the adrenal cortex and not merely a secondary compensation for DOC-induced sodium retention, for it was observed in every instance, whether or not DOC was concomitantly being secreted and whether or not sodium retention occurred. Conceivably, a decrease in aldosterone secretion might also occur in part as an *indirect* effect whenever SU-4885-induced secretion of DOC results in significant sodium retention.

In order to study the effects of SU-4885 upon aldosterone secretion it was necessary to establish conditions that would preclude the secretion of large amounts of DOC in response to SU-4885. At this point advantage was taken of the fact that

aldosterone secretion and DOC secretion are regulated through different mechanisms. DOC secretion is under the influence of ACTH, whereas aldosterone secretion is regulated by an extrapituitary mechanism (15). When sufficient amounts of ACTH-suppressing steroids such as prednisone or Dexamethasone® were given, DOC secretion was precluded; aldosterone secretion remained unaffected, however, and it became possible to study the effect of the 11β-hydroxylase inhibitor upon aldosterone secretion as an isolated phenomenon.

It is noteworthy that inhibition of aldosterone secretion by SU-4885 caused sodium loss and potassium retention in a normal individual on a *liberal* sodium diet (Figure 4) as well as in those on low sodium diets. The thesis that aldosterone may have an appreciable effect upon renal function in the normal individual on liberal sodium intake has found independent support in studies with the steroid-17-spirolactones (16). These "aldosterone antagonists" induce sodium loss only in the presence of aldosterone or aldosterone-like steroids (17). They are without effect in patients with untreated Addison's disease but have appreciable effect in normal individuals or patients with Addison's disease who are pretreated with DOC. The effects are most conspicuous in individuals who are secreting large amounts of aldosterone but are also appreciable in normal individuals in whom only small amounts of aldosterone are being secreted owing to a liberal intake of sodium.

In patients with secondary hyperaldosteronism associated either with dietary sodium restriction or pathological "edema-forming" states, SU-4885 had little effect upon sodium or potassium excretion in the absence of ACTH suppression. Presumably the inhibition of aldosterone synthesis did not, under these conditions, lead to sodium diuresis because large quantities of DOC were secreted in response to SU-4885. The concurrent administration of ACTH-suppressive steroids with SU-4885 allowed aldosterone inhibition to predominate and led to sodium diuresis and potassium retention (Figures 5, 7).

The failure of sodium diuresis to occur after SU-4885, even with ACTH suppression, in the patient with primary hyperaldosteronism is of considerable interest. This patient also responded to the steroid-17-spirolactones with conspicuous potassium retention and relatively little sodium diuresis, whereas most individuals show principally sodium diuresis in response to the "aldosterone antagonists."

SUMMARY

1). 2-Methyl-1,2-bis-(3-pyridyl)-1-propanone (SU-4885) is a potent inhibitor of 11β-hydroxylation of steroids in man.

2). As a result, SU-4885 interferes with adrenocortical synthesis and secretion of three major adrenal steroids—cortisol, corticosterone and aldosterone.

3). Since cortisol controls adrenocorticotropic hormone (ACTH) release, administration of SU-4885 gives rise to a compensatory increase in ACTH.

4). In the presence of inhibition of 11β-hydroxylation, ACTH promotes secretion of desoxycorticosterone (DOC) and 11-desoxycortisol, the former a potent mineralocorticoid.

5). The net effect of SU-4885 on electrolyte excretion is the algebraic sum of its DOC-promoting and aldosterone-inhibiting activities.

ACKNOWLEDGMENT

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STUDIES ON ISOCITRIC AND LACTIC DEHYDROGENASES IN EXPERIMENTAL MYOCARDIAL INFARCTION * †

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(Submitted for publication June 2, 1959; accepted June 19, 1959)

It was the purpose of this investigation to attempt to explain the curious pattern of serum enzyme activity which has been seen clinically following myocardial infarction. This pattern, which consists of fairly uniform elevations of the activity of lactic dehydrogenase (LDH) (1–3), glutamic-oxaloacetic transaminase (GOT) (4–6) and aldolase (1), contrasts sharply with the failure to demonstrate increased activity of isocitric dehydrogenase (ICD) (6–8). Since myocardium has been reported rich in ICD activity (7, 9), and since it has been assumed that the increase in serum enzyme activity following myocardial infarction has been related to the release of the enzyme contents of the damaged tissue (10, 11), it appeared that ICD either failed to leave the damaged cells or, if it were liberated, that it had a fate in serum different from that of LDH or GOT. The elevation of serum ICD activity in liver disease has been well documented (7, 8), and therefore the failure to demonstrate increased ICD activity after myocardial infarction was even more striking.

The investigation to be reported was designed to provide answers to the following questions. *1*) Is ICD released into the blood after myocardial infarction? *2*) What is the fate of ICD and of LDH in blood following myocardial infarction? *3*) At what rates are exogenously administered enzymes cleared from the blood of normal animals? *4*) Is the rate of "clearance" altered following hepatectomy or nephrectomy? Experiments were divided into two parts: *1*) the production of experimental myocardial infarctions in dogs, with the study of serum activity of ICD and LDH in coronary sinus and vena cava blood, and comparison of the activity of these enzymes in normal and infarcted myocardium; *2*) a study of the rates of "clearance" of ICD and LDH after intravenous injection in normal and in hepatectomized and nephrectomized dogs.

METHODS

General procedures. Unselected mongrel dogs, weighing between 10 and 18 Kg., were used. All surgical procedures were performed under pentobarbital sodium anesthesia (30 mg. per Kg.) and dogs employed in experiments requiring thoracotomies received oxygen under positive pressure.

Blood samples for enzyme assay were drawn through No. 10 French siliconized, polyethylene catheters. Each sample was centrifuged and the serum was carefully separated and recentrifuged to insure that no cells remained as contaminants. Sera were frozen immediately following centrifugation and the enzyme activity was assayed within one week of the time of collection. ICD was assayed using the method of Wolfson and Williams-Ashman (12), and activity was expressed as millimicromoles of triphosphopyridine nucleotide reduced per milliliter of serum per hour. LDH activity was determined with a modification of the method of Hill and Levi as described previously (1), and activity was expressed as the change in optical density per 0.01 ml. of serum per 30 minutes. The error of the determination of enzyme activity as based on duplicates of the same sample is about 5 per cent for ICD and about 2 per cent for LDH.

Open chest myocardial infarction procedure. Employing a left lateral thoracotomy incision, a catheter was guided into the coronary sinus via the right external jugular vein. The catheter was secured with 4–0 silk to the wall of the coronary sinus. A second catheter was guided into the superior vena cava through the left external jugular vein. The circumflex branch of the left coronary artery was exposed and dissected free. This branch was selected for ligation, because its occlusion produces a homogeneous infarct in the posterior papillary muscle (13). Homogeneous infarcts were desired to permit accurate appraisal of enzyme activity in infarcted myocardium.

Six dogs were subjected to coronary ligation at the time of thoracotomy. Ligatures were placed within 1

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† This paper is the sixth in a series of investigations on serum enzymes.

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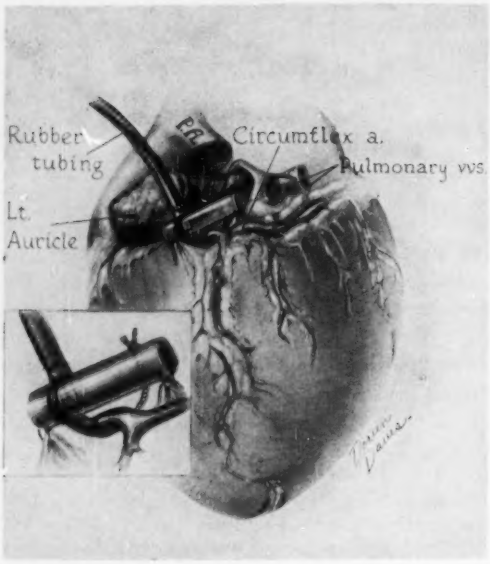

FIG. 1. APPARATUS EMPLOYED IN THE PRODUCTION OF CLOSED CHEST MYOCARDIAL INFARCTIONS

The apparatus consisted of a 2 cm. segment of polyethylene tubing attached at right angles to a rubber tube of sufficient length to extend from the heart to the chest wall. Tightening the nylon loop compressed the artery against the base of the polyethylene tube, producing coronary occlusion.

cm. of the coronary ostia. Five dogs developed ventricular fibrillation shortly after ligation; however, three of them were successfully defibrillated with electric shock. One dog, which developed acute heart failure and died four hours after ligation, was discarded. Dogs were followed continually with electrocardiograph recordings. Within an hour after ligation the chests were closed and the animals were permitted to recover. Samples were taken from the coronary sinus and vena cava following infarction at 2.5, 8, 16, 24, 36 and 48 hours. Thereafter, samples were drawn every 24 hours until the dogs died or were sacrificed.

Closed chest myocardial infarction procedure. Closed chest myocardial infarctions were performed on four dogs. After the circumflex artery was exposed, a loop of No. 2 braided nylon was placed around the artery about 3 cm. from the ostium and threaded into the infarction apparatus illustrated in Figure 1. The apparatus was secured to the myocardial fat pads with 4–0 silk, and the rubber tube containing the long end of the nylon loop was brought out through the chest wall and secured. This apparatus effectively produced occlusion in all of the animals studied and prevented the breaking of ligatures and tearing of lungs and pleural adhesions which have sometimes complicated closed chest myocardial infarctions produced by the overhand knot technique (11, 14). Coronary sinus and vena cava catheters were filled twice daily with sodium heparin to prevent clotting. When the enzyme activity returned to preoperative levels, usually within five days, the dogs were anesthetized as previously described and placed on positive pressure oxygen. Five minutes prior to tightening the nylon loop an intravenous infusion was started. This infusion, which contained papaverine hydrochloride, 8 to 10 mg. per Kg. diluted in 50 ml. of normal saline, was administered at a constant rate over a 10 minute period. According to the studies of McEachern, Smith and Manning, papaverine hydrochloride decreases significantly the mortality rate following circumflex artery occlusion in the dog (15). All dogs were followed with electrocardiograph tracings and serial blood samples were drawn as described above. Two dogs developed ventricular fibrillation and died. Atrial fibrillation was treated with lanatoside C; ventricular tachycardia was treated with procaine amide.

Myocardial enzyme assays. Assays of enzyme activity in myocardial tissue were performed on homogenates of tissue. Weighed samples of infarcted and normal myocardium were finely minced, and homogenized in a Potter-Elvejem apparatus as described by Meister (16). After centrifugation at 5,000 G for 60 minutes, enzyme assays were performed on the supernatant. Activities were expressed in terms of protein content, and the protein content was determined by the quantitative turbidimetric method of Bossak, Rosenberg and Harris (17).

Hepatectomy procedure. Total hepatectomies were performed on five dogs, employing a modification of the procedure described by Firor and Stinson (18). A thoracoabdominal approach was employed as it offered better exposure than the midline incision originally described. A siliconized glass "Y" tube was utilized to bypass the liver. Prior to dividing the portal vein, 5 ml. of 1 to 500,000 epinephrine was injected intraportally to minimize hepatic congestion, thereby decreasing the blood loss during hepatectomy. McMichael demonstrated that liver volume could be reduced by as much as 50 per cent following the intraportal injection of epinephrine (19).

Nephrectomy procedure. Bilateral nephrectomies were performed on two dogs. A midline abdominal incision was used to avoid cutting muscles and the consequent liberation of enzymes into the circulation. The kidneys were exposed and, after the pedicles were ligated and divided, the kidneys were removed.

Injection studies. With the exception of the portal vein studies, enzymes were injected through a polyethylene catheter into the vena cava. Portal vein injections were administered through a small catheter threaded into the portal vein through a mesenteric vein. In dogs, Sigma [1] ICD, 0.4 to 0.7 mg. per Kg., and NBC [2]

[1] The isocitric dehydrogenase used in these experiments was extracted from pig hearts by the Sigma Chemical Company of St. Louis, Mo., and was provided through the courtesy of Mr. Dan Broida.

[2] The lactic dehydrogenase was extracted from rabbit hearts by the Nutritional Biochemicals Corporation of Cleveland, Ohio.

TABLE I

Open chest myocardial infarction

Time	Dog. No. 27 (15.5 Kg. male)				Dog No. 28 (12.6 Kg. female)				Dog No. 30 (13.2 Kg. male)			
	Coronary sinus		*Vena cava*		Coronary sinus		*Vena cava*		Coronary sinus		*Vena cava*	
	ICD*	LDH*	ICD	LDH	ICD	LDH	ICD	LDH	ICD	LDH	ICD	LDH
Preinfarction	160	45	160	46	160	68	165	71	135	50	135	48
Postinfarction:												
2.5 hrs.	240	48	215	47	260	171	220	168	335	140	255	143
8 hrs.	475	53	300	50	970	287	325	283	610	300	455	300
16 hrs.	650	126	430	127	Died				1,230	438	865	430
24 hrs.	680	148	650	141					1,360	504	1,220	452
48 hrs.	1,080	170	1,080	154							Died	
76 hrs.	970	70	920	39								
96 hrs.	190	44	180	38								
124 hrs.	215	38	200	36								
150 hrs.	240	34	240	32								

* ICD = isocitric dehydrogenase; LDH = lactic dehydrogenase.

LDH, 0.1 to 0.2 ml. per Kg., were injected simultaneously over 10 minute intervals. Serial samples were drawn at 2, 10, 20, 40, 60, 90, 180, 240 and 420 minutes.

Several rabbits were studied to determine the normal "clearance" curves of ICD and LDH in a species other than the dog. The ICD employed was extracted from rabbit hearts and preserved by lyophilization according to the method of Ochoa (20).

RESULTS

Myocardial infarction studies

Table I and Figure 2 record the data obtained from the open chest experiments. That enzymes are released following surgical trauma has been well documented (14, 21, 22). It has been dem-

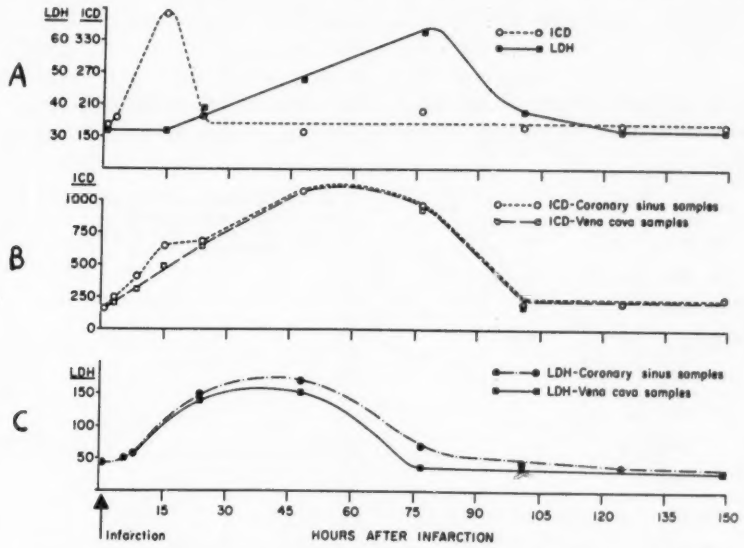

FIG. 2. ENZYME ACTIVITY AFTER OPEN CHEST MYOCARDIAL INFARCTION IN DOG No. 27

A. Differences between coronary sinus and vena cava enzyme activity after myocardial infarction. The graph is based on coronary sinus enzyme activity minus vena cava enzyme activity, using normal levels as a baseline. LDH = lactic dehydrogenase; ICD = isocitric dehydrogenase. B. ICD activity in vena cava and coronary sinus after myocardial infarction. C. LDH activity in vena cava and coronary sinus after myocardial infarction.

TABLE II

Closed chest myocardial infarction

Time	Dog. No. 42 (10.0 Kg. male)				Dog No. 44 (14.5 Kg. male)			
	Coronary sinus		*Vena cava*		Coronary sinus		*Vena cava*	
	ICD*	LDH*	ICD	LDH	ICD	LDH	ICD	LDH
Normal	120	94	115	92	115	80	110	74
Postinfarction:								
1 hr.	120	90	110	88				
3 hrs.	220	85	160	83	215	76	160	70
6 hrs.	310	228	215	142	325	135	190	125
10 hrs.	670	500	540	424	300	185	270	155
16 hrs.	500	420	325	340	215	250	110	220
24 hrs.	120	310	110	198	110	166	110	125
44 hrs.	115	100	110	88	120	80	115	78
52 hrs.					100	82	110	75
72 hrs.	110	92	100	90				
96 hrs.	120	88	110	84				

* See footnote, Table I.

onstrated that pentobarbital sodium anesthesia has no effect on serum enzyme activity (*22*). We observed increases in both ICD and LDH activity following thoracotomy; however, the increased activity of both these enzymes was similar in vena cava and coronary sinus samples. The elevations in enzyme activity illustrated in Figures 2B and 2C were the result of thoracotomy as well as of myocardial infarction. The period during which coronary sinus enzyme activity was greater than vena cava activity revealed the time during which the enzymes were being released from the injured myocardium. Figure 2A demonstrates the periods during which ICD and LDH were being released as determined by differences between coronary sinus and vena cava enzyme activity.

To eliminate the confusing effect of the enzymes released as a result of surgery, closed chest myocardial infarctions were performed. The results of these experiments are illustrated in Figure 3 and Table II. Elevations of serum ICD activity occurred within three hours following coronary ligation and returned to normal limits within 24 hours. Increases in the activity of LDH were

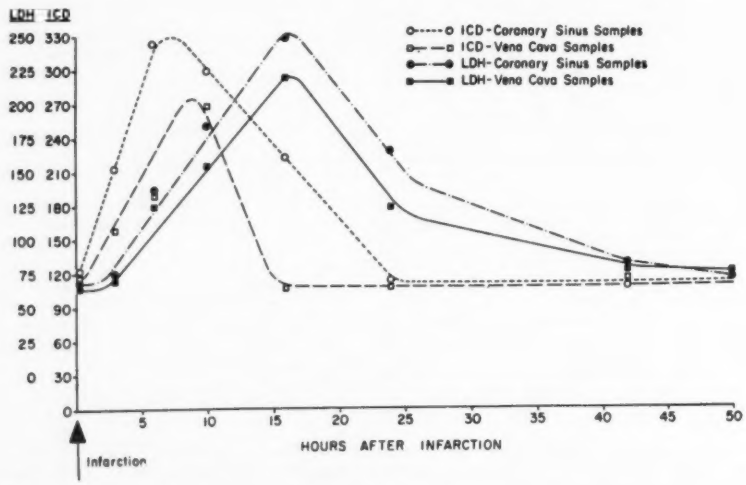

FIG. 3. ENZYME ACTIVITY AFTER CLOSED CHEST MYOCARDIAL INFARCTION IN DOG No. 44

LDH and ICD activity in vena cava and coronary sinus after myocardial infarction.

TABLE III

Enzyme activity in infarcted and normal myocardium of five dogs

Tissue	ICD*	LDH†
Normal myocardium	20.3–27.0	23.1–32.3
Infarct. myocardium	1.8– 9.0	3.1–13.2

* Isocitric dehydrogenase expressed as millimicromoles triphosphopyridine nucleotide reduced per minute per milligram protein at 25° C.

† Lactic dehydrogenase expressed as micromoles diphosphopyridine nucleotide oxidized per minute per milligram protein at 38° C.

not observed until six hours after infarction; however, elevated activity was maintained for about 44 hours. These data are illustrated in Figure 3, which demonstrates the rapid rise and fall of ICD in comparison to LDH and confirms the open chest infarction profile illustrated in Figure 2A.

Myocardial enzyme assays

The enzyme activity in the homogenates prepared from normal and infarcted myocardium is recorded in Table III. The infarcted tissue contained less ICD and LDH activity than the normal myocardium.

Enzyme clearance studies

Figure 4 summarizes the findings of these studies. Injections of ICD and LDH into normal dogs and rabbits demonstrated the disappearance of exogenous ICD within 90 minutes following its injection; LDH activity was not observed to return to normal levels until about seven hours after injection.

Injections into the portal vein did not produce "clearance" rates different from those observed

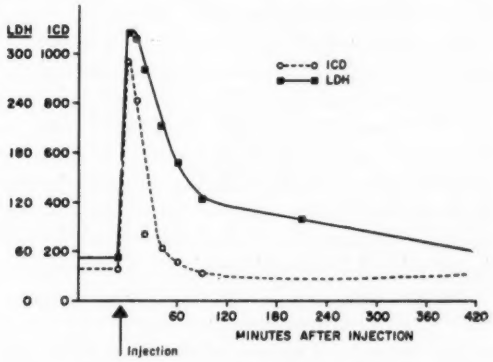

FIG. 4A. VENA CAVA ENZYME INJECTION

Rate of enzyme fall after the injection of 68.8 mg. ICD and 1 ml. LDH into the inferior vena cava of Dog No. 29.

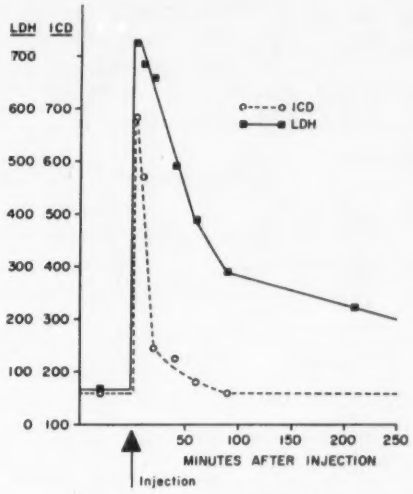

FIG. 4B. PORTAL VEIN ENZYME INJECTION

Rate of enzyme fall after the injection of 58 mg. ICD and 2.9 ml. LDH into the portal vein of Dog No. 27.

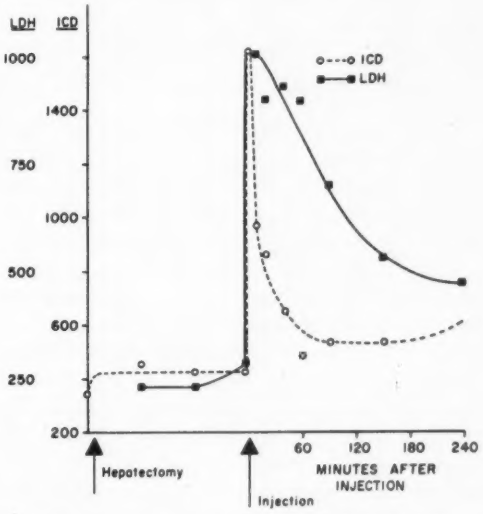

FIG. 4C. ENZYME INJECTION AFTER HEPATECTOMY

Rate of enzyme fall after the injection of 85.4 mg. ICD dehydrogenase and 2.4 ml. LDH into the inferior vena cava four hours after completion of total hepatectomy in Dog No. 31.

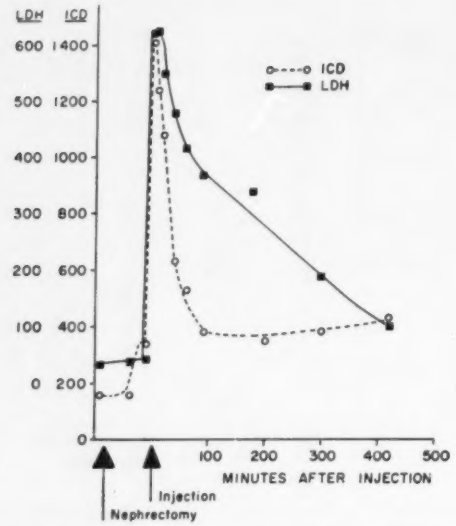

FIG. 4D. ENZYME INJECTION AFTER NEPHRECTOMY

Rate of enzyme fall after injection of 60 mg. ICD and 1.5 ml. LDH into the inferior vena cava two hours after completion of bilateral nephrectomy in Dog No. 34.

in the normal preparations. Injections following hepatectomy and nephrectomy also produced similar disappearance curves.

DISCUSSION

Myocardial infarction studies

These data document the release of ICD following myocardial infarction. ICD was released earlier than LDH, but ICD activity remained elevated for less than 24 hours; by contrast LDH activity remained elevated for at least 44 hours.

The coronary sinus enzyme levels are pertinent to previous discussions concerning the source of increased serum enzyme activity following myocardial infarction (11, 21). Following infarction, the activity of samples drawn from the coronary sinus was consistently greater than was the activity in samples drawn from the peripheral circulation. This difference corroborates the findings of Ruegsegger, Nydick, Freiman and LaDue (11) and indicates that the anoxic myocardium is responsible for a significant portion, if not all, of the liberated enzyme. It is important to note that, when the coronary sinus activity was no longer greater than the activity found in the vena cava, the coronary sinus and vena cava enzyme activities had both returned to normal. This indicates that the observed elevations were primarily a direct result of enzyme release from the myocardium and not the result of a nonspecific response to trauma (21).

Elevation of serum LDH activity was not observed until six hours after coronary ligation. This delay prior to the release of LDH confirms the findings of Jennings, Kaltenbach and Smetters (23) and Ruegsegger and associates (11).

Myocardial enzyme assays

Several studies have previously demonstrated that homogenates prepared from infarcted myocardium contain less GOT, LDH, glutamic-pyruvic transaminase and succinic dehydrogenase activity than homogenates prepared from normal myocardium (11, 23). Our studies demonstrate that the activity of ICD in the infarcted tissue is similarly reduced. This observation along with the finding of increased ICD activity in coronary sinus blood after myocardial infarction is strong evidence that ICD is released from damaged myocardium.

Enzyme clearance studies

In these experiments sufficient amounts of enzyme were injected to produce elevations of activity which would be within the range observed in disease states. With ICD activity elevated to seven times normal, "clearance" was complete within 90 minutes. With similar elevations LDH was "cleared" in about seven to 10 hours.

The serum activity of LDH and GOT following experimental myocardial infarction has been found to correlate closely with the activity of these enzymes following myocardial infarctions in human beings (14, 24). If the activity of serum ICD after experimental infarction also parallels the clinical picture, the early and transient elevation of ICD explains the clinical failure to observe increased activity after infarction. The rapid disappearance of serum ICD activity following injection explains, at least in part, the transitory nature of the elevation which is observed after experimental myocardial infarction.

In view of the clinical finding that ICD has been reported elevated only in diseases involving

the hepatic parenchyma (7, 8), the liver was considered first as a possible site of ICD inactivation. However, the similar disappearance curves of ICD and LDH observed following vena cava injections, intraportal injections and injections in hepatectomized dogs led us to reject this hypothesis. One may thus infer that the elevation of serum ICD activity observed in liver disease is a result of the continuous liberation of ICD from damaged cells rather than a result of impaired hepatic inactivation of the enzyme.

The normal disappearance curves in nephrectomized and hepatectomized dogs in conjunction with the data obtained following intraportal injections indicate that the liver and kidney are not responsible for "clearing" ICD and LDH from the circulation.

Numerous investigators have commented on the speed of release of enzymes from damaged cells and on the rate of "clearance" of enzymes following injection into the blood stream (6, 10, 14). One of the variables considered in this respect has been enzyme molecular weight. Therefore a comparison of molecular weight with rate of "clearance" is of interest. The molecular weight of ICD has been reported as 61,000 (25), LDH as 135,000 (26) and GOT as 60,000 (27). Wroblewski injected homogenates of myocardium into dogs and observed similar disappearance curves for GOT and LDH over a period of four hours (10). His LDH "clearance" was comparable to that observed in our studies (10, 24). As noted above, ICD "clearance" was more rapid than LDH; however, comparison of the molecular weights of ICD, LDH and GOT with their rates of disappearance following injection demonstrates that although ICD and GOT have similar molecular weights, ICD is "cleared" more rapidly than GOT. On the other hand, LDH and GOT with similar rates of disappearance have different molecular weights. If the weights reported coincide with those of the enzymes used in these experiments, molecular weight cannot explain the differences observed in rate of "clearance."

SUMMARY

Isocitric dehydrogenase (ICD) is released from injured myocardium following myocardial infarction. ICD is released shortly after infarction and for only a brief period of time; by contrast, lactic dehydrogenase (LDH) is released later than ICD and remains elevated longer.

ICD is rapidly "cleared" from the circulation following injection whereas LDH "clearance" is more protracted.

ICD and LDH are apparently not "cleared" by any single organ. The injection of these enzymes into the vena cava and portal vein of dogs and into the vena cava of hepatectomized and nephrectomized dogs demonstrates similar "clearance" rates.

ICD activity is transiently elevated during the first 24 hours following experimental myocardial infarction. The brief duration of this elevation is apparently due to the extremely rapid "clearance" of ICD and to the brief period of its liberation. If this brief period of elevation observed experimentally also occurs in patients with myocardial infarction, it presumably explains the clinical failure to demonstrate elevated levels of this enzyme after infarction.

ACKNOWLEDGMENT

We are indebted to Dr. Roy B. Cohn and Dr. Norman E. Shumway for their suggestions on surgical technique, to Dr. Raymond C. Stofer for his help and cooperation in the surgical laboratory, and to Mr. Charles J. Lewin and Mr. Donald R. Johnson for their suggestions and criticisms.

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STUDIES OF COLON CONTRACTILITY IN DOGS. I. DESCRIPTION OF METHOD AND EFFECT OF PARASYMPATHOMIMETIC AGENTS AND SEROTONIN * †

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The effects of autonomic substances and 5-hydroxytryptamine (serotonin) upon the smooth muscle of the intestine are not completely understood despite extensive experimentation *in vitro* and *in vivo*. Serotonin may play an important role in the normal regulation of intestinal motility, since, *in vitro*, it causes strong contraction of intestinal smooth muscle (1, 2); when given intravenously both to man and certain experimental animals, it stimulates intestinal motility (3–5); and its excess production is associated with diarrhea and increased peristalsis in the metastatic carcinoid syndrome (6, 7). The major body depot of serotonin is the gastrointestinal tract where it is localized in the enterochromaffin cells of the mucosa. Its mechanism of action upon the intestine is unclear, and there are many conflicting reports on its relationship to the autonomic nervous system (1–5, 8–10).

In order more closely to ascertain the effect of acetylcholine, methacholine and serotonin upon mammalian intestinal smooth muscle, an *in vivo* experimental technique has been devised. This method, which is easy to perform and yields reproducible results, makes it possible to introduce the test substances directly and initially into the intact intestine. This is accomplished by injections into the terminal or end artery, which nourishes a segment of proximal colon, of an anesthetized dog.

The purpose of the present report is to describe the method, to detail the effect of lowering tissue cholinesterase upon the action of parasympathomimetic agents, and to help clarify the mechanism of action of serotonin, with particular reference to the parasympathetic nervous system.

METHODS AND MATERIALS

Mongrel dogs weighing 10 to 20 Kg. were used in all experiments. The animals were fasted for 48 hours prior to the study to insure an empty gastrointestinal tract. Intraperitoneal injection of 0.5 ml. per Kg. Dial® with Urethane [1] induced a surgical plane of anesthesia within 15 minutes, which was maintained for eight to 10 hours. An endotracheal tube was inserted and attached to a positive pressure oxygen breathing apparatus in order to insure adequate oxygenation. A constant intravenous drip of 5 per cent glucose solution was given slowly throughout. Although aseptic surgical technique was not employed, the operators wore rubber gloves during the preparation of the specimen and maintained a clean and relatively bloodless field. Following a midline or right rectus incision, the mesenteric arterial blood supply to the proximal 7 to 12 cm. of colon was isolated. A distal branch of the artery was injected with 1.0 μg. of acetylcholine, and the segment of proximal colon in which spasm occurred was marked and then intubated with a rubber catheter which was passed through the terminal ileum and ileocaecal valve into the segment. The distal end of this catheter was attached to a small latex balloon approximating the size of the bowel lumen but not distending it. Balloon, catheter, and an attached manometer were all filled with water, and this system was connected by air-filled tubing to a writing arm which recorded contractions kymographically (Figure 1). Throughout all experiments the exposed loop of gut was kept moistened with warm Locke's solution.

To facilitate injections into the end artery, a short length of silk was passed under the vessel so that it could

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[1] Generously supplied by Dr. Robert R. Smith, Ciba Pharmaceutical Products, Summit, N. J. Dial®-Urethane contains diallyl-barbituric acid, 10 per cent; Urethane, 40 per cent; monoethylurea, 40 per cent; water, 10 per cent.

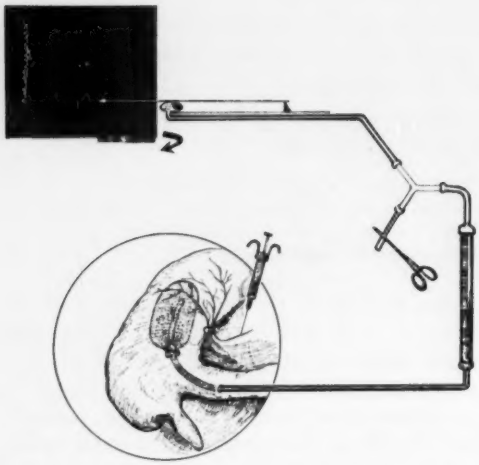

FIG. 1. DIAGRAM OF THE INTUBATED SEGMENT WITH ISOLATED TERMINAL ARTERY INTO WHICH TEST AGENTS ARE INJECTED

Balloon, catheter and variable length of manometer only are water filled.

be stabilized by exerting tension prior to each injection. All injections were made with a 1 ml. syringe and a 27 gauge needle (Figure 1). Injections were noted on the recording drum by means of an electric signal marker or manual compression of the rubber tubing leading from the manometer. Unless otherwise specified, all test substances [2] were injected in volumes of 0.1 ml. as rapidly as possible, each injection averaging one second. All test solutions were freshly prepared for each experiment and dissolved in Locke's solution containing bicarbonate.[3] The pH of this solution ranged from 7.5 to 7.7. Injection of this solution was without effect upon the intestine.

Great care was exercised during the preparation of the di-isopropylfluorophosphate (DFP) solution.[4] The material was always handled under a hood, the experimenter's arms extending through aperatures in its window. One-tenth gram DFP (0.1 ml.) was carefully withdrawn from the stock container, placed in a 100 ml. volumetric flask, and diluted with buffered Locke's solution, yielding a final concentration of 1.0 mg. per ml. The container was then carefully sealed and immersed in a metal can of sodium bicarbonate. All DFP injections were given within one-half hour of preparation.

Cholinesterase determinations were performed on segments of colon in a number of experiments. Since the test segment of colon could not be used as its own control for cholinesterase content, a segment distal to it was also removed at the end of some experiments in which DFP had been given. Cholinesterase values for these two sections of colon were comparable under normal circumstances. Determinations of tissue cholinesterase in these segments were performed by the Warburg manometric technique with acetylcholine as substrate (11). The average of three 10 minute incubations was chosen as the final value, and the results were expressed as microliters of CO_2 per 0.1 Gm. tissue. Control determinations in 30 animals revealed that the normal cholinesterase value varied between 65 and 125 μl. CO_2 per 0.1 Gm. tissue, with an average of 100. All determinations were performed immediately or after freezing (11).

[2] Serotonin kindly provided as Serotonin Creatinine Sulfate by Dr. D. A. Sheperd, Upjohn Company, Kalamazoo, Mich.

[3] One L. Locke's solution to 2.1 Gm. sodium bicarbonate.

[4] Kindly provided by Dr. Bernard J. Jandorf, Army Chemical Center, Md.

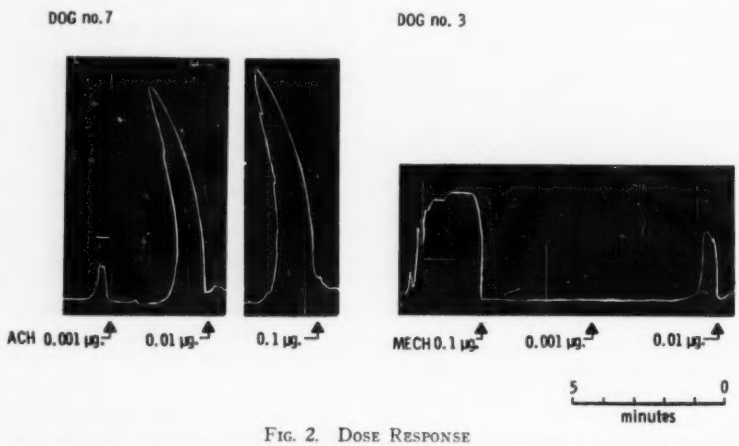

FIG. 2. DOSE RESPONSE

Note characteristic responses for acetylcholine (ACH) and methacholine (MECH). Read right to left.

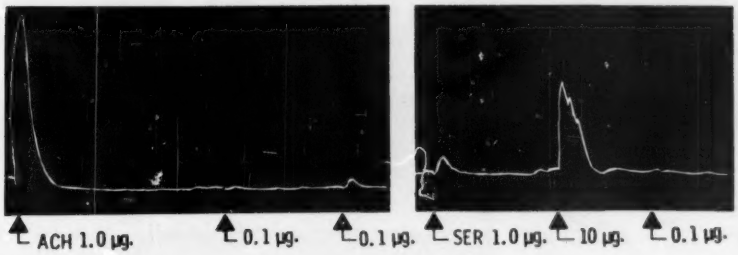

FIG. 3. COMPARISON OF ACETYLCHOLINE (ACH) AND SEROTONIN RESPONSES

Note that character of relaxation of serotonin (SER) differs from that following ACH and is irregular. Read left to right.

In discussing our results, we define any decrease in threshold dose of a test substance (decrease in dosage level required to produce a discrete contraction of the bowel) as *potentiation*, whereas a heightened and/or prolonged response at the same dosage level is termed *augmentation*.

In all experiments, there was little or no baseline activity, and threshold responses were easily identified and always reproducible in the same animal.

RESULTS

Effects of acetylcholine, methacholine and serotonin

In 30 experiments the amount of acetylcholine required to produce minimal discrete contractions averaged 0.01 μg. (range, 0.001 to 0.1 μg.), and in 15 animals the mean threshold dose for methacholine was 0.1 μg. (range, 0.001 to 1.0 μg.).

While the ratio for the average threshold doses of acetylcholine/methacholine was 10/1, this relationship varied considerably and unpredictably in the individual experiment. A characteristic response was noted for each of these substances, contractions caused by acetylcholine being of shorter duration than those caused by methacholine. In 26 animals, serotonin caused a vigorous intestinal contraction with the average threshold dose of 0.01 μg. (range 0.001 to 1.0 μg.). While the mean threshold dose of serotonin was the same as that for acetylcholine, the character of the curve of relaxation differed slightly from that of acetylcholine. The response curves for each of these substances are shown in Figures 2 and 3.

Effect of inhibition of colon cholinesterase by DFP

Tissue cholinesterase in the intubated segment was abruptly reduced following intra-arterial injection of DFP. Amounts of this drug in excess of 50 μg. per Kg. could not be used because they induced prolonged colonic spasm which prevented retesting for several hours. Although spasm was noted following administration of 25 to 50 μg. per Kg. intra-arterially (Figure 4), repeat injections invariably could be carried out within an

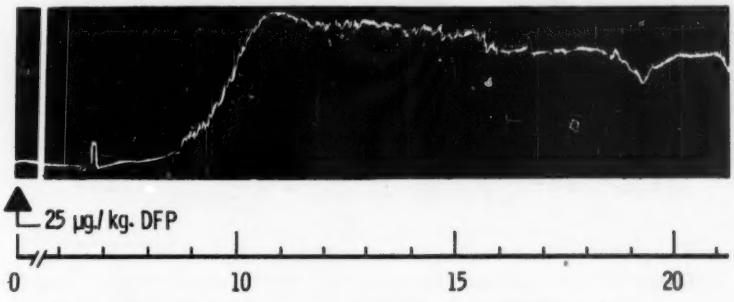

FIG. 4. EFFECT OF DI-ISOPROPYLFLUOROPHOSPHATE (DFP) ON MOTILITY

Duration of this spasm induced by intra-arterial injections of 25.0 μg. per Kg. DFP was 30 minutes, after which test substances could be administered. Read left to right.

TABLE I

Effect of inhibition of colon cholinesterase (CHE) on response to acetylcholine (A), methacholine (M)

Dog	Drug	Control threshold dose	DFP*	Post-DFP threshold	Augmentation	Inhibition colon (CHE)
No.		*μg.*	*μg./Kg.*	** μg.*		*%*
4	A	0.1	50	0.0001	+	91†
	M	0.01		0.01	+	
7	A	0.1	50	0.1	+	90†
	M	0.1			+	
8	A	0.1	50	0.01	+	80†
	M	1.0		0.1	+	
9	A	0.01	50	0.1	+	50†
	M	0.001		0.01	+	
14	A	0.1	50	0.1	+	67
	M	1.0			+	
15	A	0.5	25	0.01	+	87
	M	0.1		0.1	+	
17	A	0.05	12.5	0.5	+	43†
	M	0.1		0.5	+	
18	A	0.05	25	0.05	+	75
	M	0.01		0.01	+	
19	A	0.5	25	0.01	+	88
	M	0.1		0.1	+	
47	A	0.5	25	0.1	+	94

* Di-isopropylfluorophosphate.

† Values are in reference to the mean cholinesterase concentrations of the colons of 30 control dogs (11).

hour. In Table I are the results of retesting the colonic segment with acetylcholine and methacholine following the injections of DFP in the range of 12.5 to 50.0 μg. per Kg. In five animals the percentage of cholinesterase inhibition is based on the ratio of cholinesterase in the test segment following DFP to that of a segment of distal colon removed at the end of the experiment. In five other animals, the ratio was in reference to the average of a control series of 30 dogs (11). It will be noted that in all instances augmentation of response was obtained (Figure 5). It is of special interest that potentiation of acetylcholine, *i.e.*, lowering of the threshold, occurred in only five animals (Nos. 4, 8, 15, 19, 47) and in all of these the inhibition of colon cholinesterase was 80 per cent or more. In the remaining animals (Nos. 7, 9, 14, 17, 18) in which potentiation of acetylcholine did not occur, cholinesterase inhibition was less than 80 per cent, except for Dog No. 7 in which it was 90 per cent. In only one experiment (Dog No. 8) did potentiation of methacholine occur.

To determine the relationship of serotonin response to the cholinergic nervous system, the com-

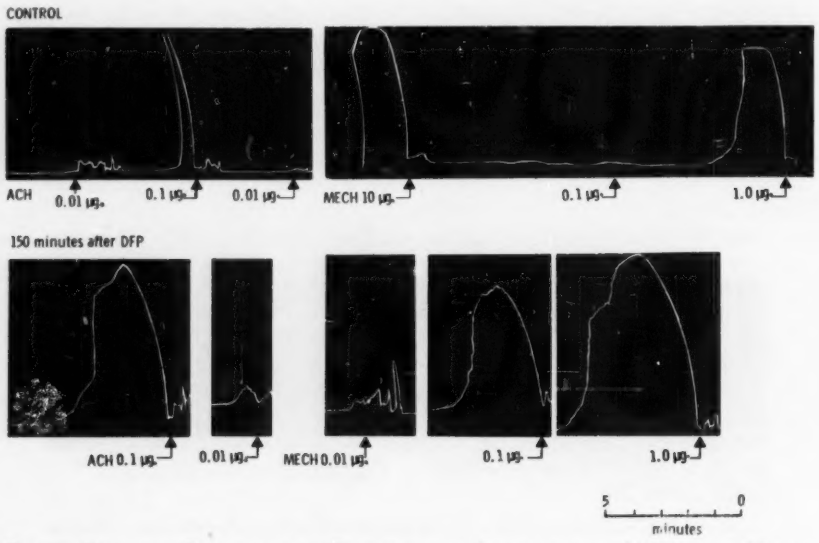

FIG. 5. EFFECT OF CHOLINESTERASE DEPRESSION ON ACETYLCHOLINE (ACH) AND METHACHOLINE (MECH) RESPONSE (DOG NO. 8)

Effect of ACH and MECH before and after injection of di-isopropylfluorophosphate (DFP), 50 μg. per Kg. Although tissue cholinesterase was inhibited 80 per cent, there was only 10-fold decrease in threshold for each substance; however, marked augmentation is seen. Read right to left.

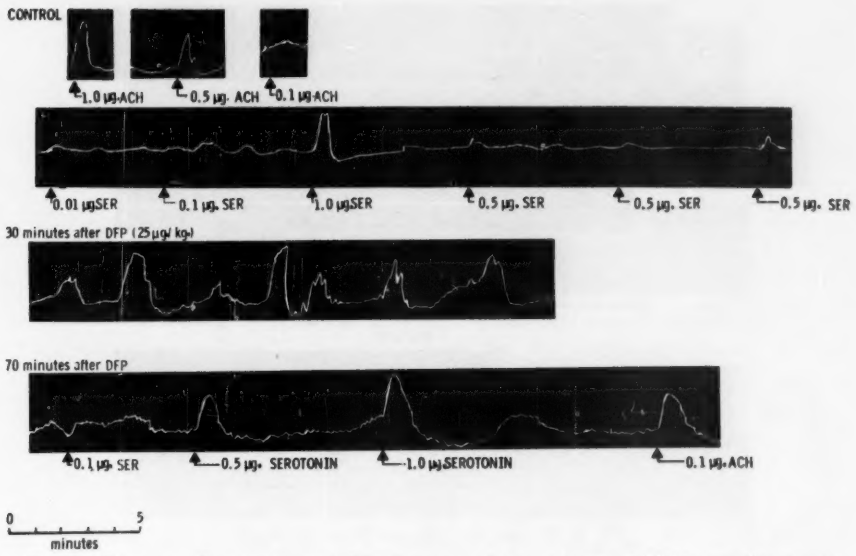

FIG. 6. EFFECT OF CHOLINESTERASE DEPRESSION ON ACETYLCHOLINE (ACH) AND SEROTONIN (SER) RESPONSE (DOG NO. 47)

Augmentation of both ACH and SER responses followed intra-arterial injection of di-isopropylfluorophosphate (DFP), 25 µg. per Kg. Change in threshold was noted also for ACH. Colon cholinesterase was reduced 85 per cent. Read left to right.

parative effect of marked lowering of colon cholinesterase (75 to 95 per cent) upon the action of serotonin and acetylcholine was studied in two animals (Nos. 47, 49). The threshold for acetylcholine decreased in one animal, but was unchanged for serotonin. Further, although serotonin response was augmented, the degree of augmentation was less than that for acetylcholine (Figure 6) and may represent an addition of effect of this agent combined with endogenous acetylcholine.

Combined action of serotonin and acetylcholine

To ascertain whether serotonin and acetylcholine enhance each other's action, we compared the effects of a mixture of half of the volume of an approximately equi-effective dose of each agent, with that of each reference dose. In no case of such combined administration did augmentation occur. In 11 of 14 observations the effects were only additive. In three trials, moreover, the response to the combination was less than that of

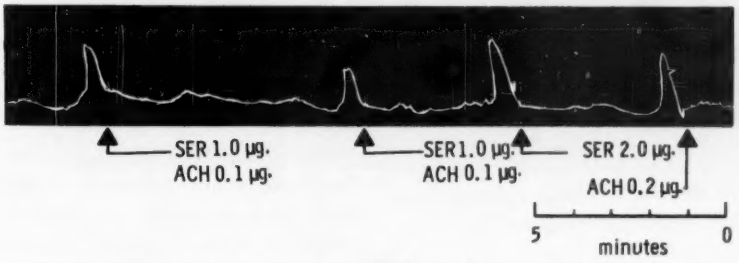

FIG. 7. COMBINED ACETYLCHOLINE (ACH) AND SEROTONIN (SER) ACTION (DOG NO. 45)

Augmentation did not result from mixture of half of an equi-effective dose of each agent, the response being merely additive on one occasion and diminished on another. Read right to left.

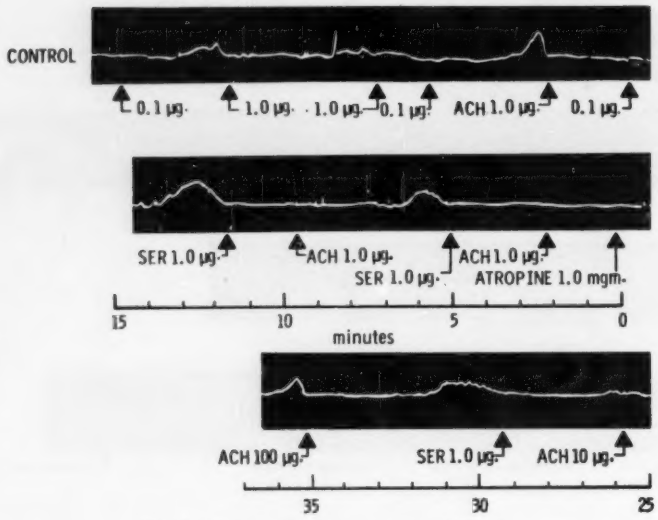

FIG. 8. EFFECT OF ATROPINE (DOG NO. 42)

Threshold dose for acetylcholine (ACH) increased 100 times after 1.0 mg. atropine, although serotonin (SER) threshold dose was unchanged; response seemed to be enhanced. Read right to left.

either reference dose (Figure 7). In 25 dogs there was no alteration of response to acetylcholine administered from five seconds to 60 minutes after serotonin.

Effect of an anticholinergic drug

The effect of an anticholinergic agent, atropine, upon the action of serotonin was observed in eight animals. Figure 8 shows the effect of 1.0 mg. of this substance on the subsequent injections of serotonin and acetylcholine. Although the threshold for acetylcholine was increased 100-fold, the serotonin threshold was unaffected and indeed in several experiments there appeared to be augmentation (Figure 8). Higher doses of atropine, 6 to 8 mg., raised the serotonin threshold 10- to 20-fold, while changing that for acetylcholine 1,000- to 5,000-fold. Even after 85 per cent inhibition of cholinesterase, the relative effectiveness of a large dose of atropine upon serotonin and acetylcholine was unchanged (Figure 9).

Ganglion blockade

Hexamethonium, 1.5 to 12.5 mg., did not block the action of either serotonin or acetylcholine; indeed, in three of four observations, it seemed to augment the serotonin response (Figure 10).

Monoamine oxidase inhibition

U-7729,[5] a methyl analog of serotonin and monoamine oxidase inhibitor (MOI), was administered in doses of 100 μg. on nine occasions in four animals. On eight occasions it produced contraction of the bowel and it augmented the response of serotonin in five of nine instances and of acetylcholine on seven of nine occasions (Figure 11). In a fifth dog, no spontaneous response to the same dose of MOI was noted, although serotonin and acetylcholine contractions were augmented. Despite a total dose of 400 μg. MOI in each of four experiments, there was no alteration in the threshold dose for either drug.

Further, administration of MOI only slightly altered the relative effect of atropine upon serotonin and acetylcholine; *i.e.*, the ratio of atropine inhibition of these agents (acetylcholine/serotonin) was between 10/1 and 100/1 (Figure 12).

[5] Supplied by Dr. J. P. Webb, The Upjohn Company, Kalamazoo, Mich.

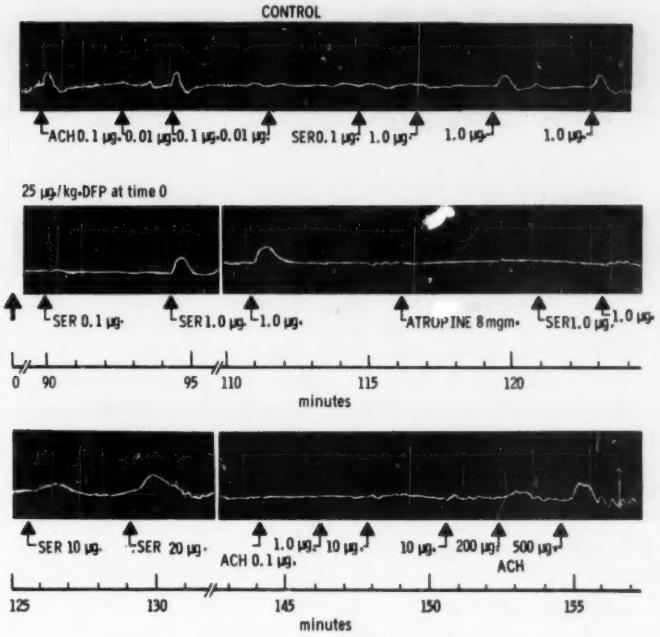
CONTROL
ACH 0.1 µg. 0.01 µg. 0.1 µg. 0.01 µg. SER 0.1 µg. 1.0 µg. 1.0 µg. 1.0 µg.
25 µg./kg. DFP at time 0
SER 0.1 µg. SER 1.0 µg. 1.0 µg. ATROPINE 8 mgm. SER 1.0 µg. 1.0 µg.
0 90 95 110 115 120
minutes
SER 10 µg. SER 20 µg. ACH 0.1 µg. 1.0 µg. 10 µg. 10 µg. 200 µg. 500 µg. ACH
125 130 145 150 155
minutes

FIG. 9. EFFECT OF DI-ISOPROPYLFLUOROPHOSPHATE (DFP) ON ATROPINE BLOCKADE (DOG NO. 49)

Depression of colon cholinesterase by DFP, 25 µg. per Kg., did not alter the relative effectiveness of a massive dose of atropine, 8.0 mg., upon acetylcholine (ACH) and serotonin (SER) responses. Colon cholinesterase was reduced 85 per cent. Read left to right.

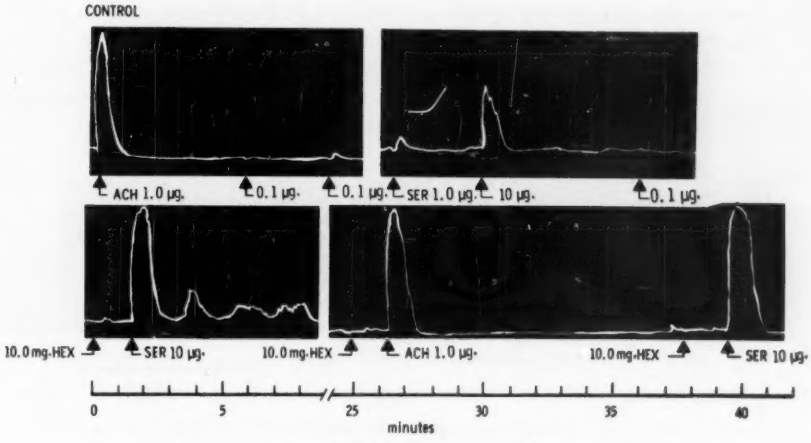

FIG. 10. EFFECT OF HEXAMETHONIUM (DOG NO. 52)

Three massive doses of hexamethonium, 10.0 mg. each, blocked neither acetylcholine (ACH) nor serotonin (SER). Effect of serotonin appeared to be augmented. Read left to right.

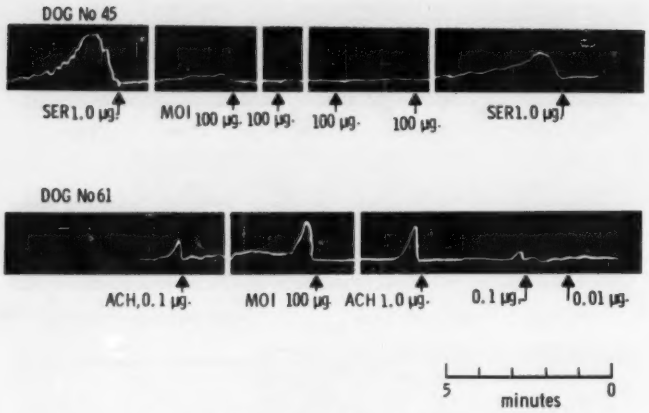

FIG. 11. EFFECT OF MONOAMINE OXIDASE INHIBITOR (MOI)

In Dog No. 45, four injections of 100 µg. MOI, U-7729, augmented the response to serotonin (SER). In Dog No. 61, 100 µg. MOI induced a contraction and augmented acetylcholine (ACH) response. Read right to left.

Antiserotonin drugs

Prochlorperazine (Compazine®) in doses of 100 µg. inhibited the action of serotonin markedly in three observations, but did not alter acetylcholine effect (Figure 13). The benzyl analog of serotonin (BAS), provided by Dr. D. W. Wooley, effected a graded inhibition of serotonin response in a dose range of 1.0 to 4.0 mg. After 1.0 mg. there was a 10-fold increase in serotonin threshold (Figure 14); after 4.0 mg., a 100-fold increase, the duration of inhibitory effect averaging about 30 minutes. When 100 mg. of BAS was given intravenously on two occasions, there occurred a 10-fold increase in threshold in one animal, but threshold was unaffected in the other. Neither prochlorperazine nor BAS changed the intestinal response to acetylcholine.

DISCUSSION

These studies on the effect of acetylcholine, methacholine and serotonin on the dog colon have demonstrated the advantages and usefulness of an *in vivo* intra-arterial injection technique. The procedure is simple, yields reproducible results and is stable enough to permit experimentation over an eight to 10 hour period. In addition, it permits recording intestinal action in a preparation that has an intact innervation and blood supply.

The contractile effect of acetylcholine and methacholine on the intestine is well known (12–

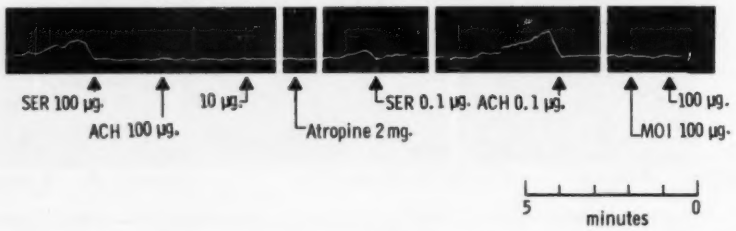

FIG. 12. EFFECT OF MONOAMINE OXIDASE INHIBITOR (MOI) ON ATROPINE BLOCKADE (DOG No. 54)

Following two consecutive injections of 100 µg. of MOI, 2.0 mg. atropine retained its relative effect upon acetylcholine (ACH) and serotonin (SER) responses. Read right to left.

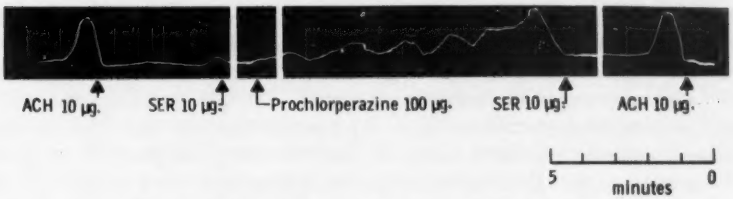

FIG. 13. EFFECT OF PROCHLORPERAZINE (DOG NO. 56)

Prochlorperazine (100 µg.) completely inhibited serotonin (SER) and did not affect acetylcholine (ACH). Read right to left.

14). In our studies the range of threshold dose of these two drugs was similar and approximates that derived from perfusion experiments (15); however, when these drugs are given intravenously, the average threshold dose range is about 1,000-fold greater (11).

Of interest is the finding that the characteristic responses to both these agents differ, the acetylcholine response being more rapid in onset and decline and the methacholine response lasting longer. The explanation of the longer methacholine response may be its slower hydrolysis by the tissue cholinesterase. The individual responses to each of these agents were reproducible over a period of eight to 10 hours, there being no tachyphylaxis or change in threshold dosage.

Study of the effect of acutely reducing the tissue cholinesterase upon the action of these agents through intra-arterial injection of DFP is limited by the reaction of the effector organ to large amounts of the anticholinesterase compound. When an amount is given sufficient to cause a greater than 90 per cent depression of cholinesterase, a sustained contraction which precludes re-injection is noted. Following this degree of cholinesterase suppression, Riker and Wescoe encountered similar spontaneous activity in a different neuroeffector system, chorda tympani nerve and the submaxillary gland of the cat (16). Further, they found that inordinate amounts of DFP were required to inactivate the remaining 10 per cent of cholinesterase. This may be attributed to the phenomenon described by Nachmansohn, Rothenberg and Feld (17) in which the reaction of cholinesterase and its inhibitor is shown to be stoichiometric but variable at different concentrations of the enzyme. At lower concentrations of cholinesterase, proportionately more DFP is required for inactivation, possibly 100,000 molecules of DFP being required for one of cholinesterase, whereas at higher concentrations, the ratio may be only 25 to 1.

That approximately 80 per cent inhibition of cholinesterase is associated with only slight (10-

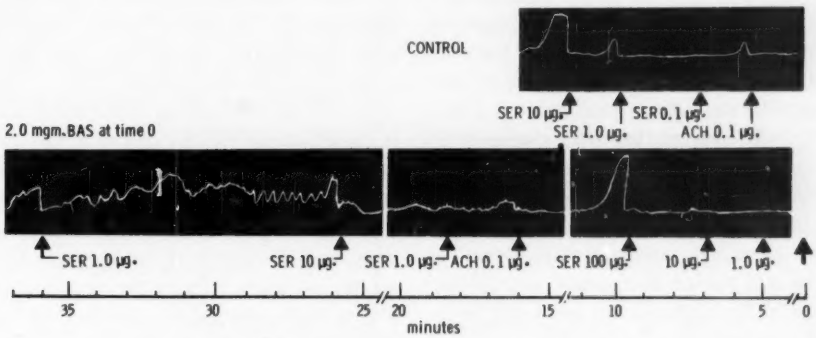

FIG. 14. EFFECT OF BENZYL ANALOG OF SEROTONIN (BAS) (DOG NO. 37)

Two mg. of BAS inhibited serotonin (SER) 100-fold for 30 minutes, but did not alter acetylcholine (ACH) response. Read right to left.

fold) alteration in threshold dosage seems surprising. However, Riker and Wescoe reported similar results in their experiments (16). Using DFP, they found that suppression of cholinesterase by at least 50 per cent was required to reduce threshold stimulus (frequency of electric shocks to the chorda tympani) and that the maximal alteration in threshold occurred only at 90 per cent suppression. When they administered supramaximal stimuli, the total response (volume of saliva) increased when cholinesterase was reduced 50 to 90 per cent; this increase was manifested principally as a prolongation of response after stimulation; the response during stimulation was altered slightly. These findings indicate that tissue cholinesterase occurs in a concentration which is far in excess of what is required for normal function.

These results in another parasympathetic-effector system are strikingly similar to those of the intestine. In the latter there was little change of threshold stimulus despite marked reduction in tissue cholinesterase and, analagous to the increased post-stimulus salivation for the submaxillary gland, there was prolongation of contraction in the intubated segment of the intestine (Figure 5). Further, Riker and Wescoe obtained the same relationship after DFP when the salivary gland was stimulated with intra-arterial injection of acetylcholine instead of by electrical shocking. These authors suggest that this augmented response is due to prolonged action of acetylcholine (or methacholine) at the myoneural junctions.

Serotonin appears to be as potent as acetylcholine in its contractile effect upon the intestinal musculature, the average threshold dose for each substance being the same and the magnitude and duration of response being comparable. There was no evidence for mutual potentiation with acetylcholine; mixtures of the two substances were at most only additive. Although the *in vitro* studies of Pick (9), using guinea pig ileum, suggested that serotonin enhanced the action of acetylcholine and related compounds, this finding is not confirmed by our work nor by that of Freyburger and associates (5).

That serotonin acts via a cholinergic pathway or mechanism seems unlikely for several reasons. First, although colon cholinesterase depression by 80 per cent or greater was associated with augmented response to both serotonin and acetylcholine, potentiation occurred only for the latter substance. Second, atropine was 100 times more potent in blocking acetylcholine than serotonin. Indeed, with lower doses of atropine, 1.0 mg. or less, serotonin response appeared to be augmented. Garven (18), using guinea pig ileum, showed that atropine had this same ratio of potency for inhibiting these two agents. Likewise, Gaddum and Hameed (1) found it only partially effective against the action of serotonin on the guinea pig ileum. However, Rocha e Silva, Valle and Picarelli (2), using a similar preparation, demonstrated that atropine was a potent inhibitor of both serotonin and acetylcholine. While Hendrix, Atkinson, Clifton and Ingelfinger (3) observed that atropine inhibited the action of serotonin upon the small intestine in man, Haverback and Davidson (4) could not confirm this finding. Since in both these experiments all substances were given intravenously, comparison with the *in vitro* and intra-arterial techniques is difficult.

That serotonin does not act via the autonomic ganglia is demonstrated by the failure of hexamethonium to block its response; indeed, an opposite effect may occur, since there was an augmented serotonin response in three of four instances in which it was given after hexamethonium. The reason for this augmentation, also suggested by Rocha e Silva and associates in their studies upon the guinea pig ileum (2), is unclear. It may be related to increased excitability of muscle fibers following ganglionic blockade, as was suggested by Feldberg (19). Possibly, large doses of methonium compounds stimulate the postsynaptic cells of the ganglia (20).

A striking observation in the experiments with hexamethonium was the failure of this agent, even in large dosage, to diminish the usual response to acetylcholine. Such diminution might have been expected since part of the action of acetylcholine upon smooth muscle of the intestine is mediated via the ganglion cells of the myenteric plexuses, and a large dose of hexamethonium may block acetylcholine at the neuroeffector junction (21). Perhaps a partial explanation, as suggested by Riker and Szreniawski (22), is that the predominant site for inhibition of acetylcholine by

hexamethonium in the ganglion is not at the postsynaptic levels, but rather at the terminals of the presynaptic fibers.

Prochlorperazine and BAS completely block the spasmogenic action of serotonin upon the intestine and do not alter the acetylcholine response. This inhibitory effect of a phenothiazine has also been shown upon the rat colon, *in vitro,* by Benditt and Rowley (23). Such behavior might be expected, since the structure of serotonin is similar to epinephrine and norepinephrine, all three substances being destroyed by monoamine oxidase, and the phenothiazines possess sympatholytic properties (24). BAS probably exerts its effects by means of a specific competition with serotonin (25).

Monoamine oxidase, which exists in high concentration in the intestine (26), plays an important role in the metabolism of serotonin. Inhibition of this enzyme augments serotonin response. The kinetics of this interaction may be analogous to that for acetylcholine and cholinesterase, since inhibition of cholinesterase is associated with an augmentation of acetylcholine effect. This interaction of serotonin and monoamine oxidase, which probably occurs at a significant rate in the intestine, may further indicate that serotonin plays a role in the regulation of motility of this organ.

Although it seems clear from these studies that exogenously administered serotonin does not act by way of a cholinergic pathway in the intact animal, it has been amply established by Bülbring and Lin (27) that this agent acts as a modulator of the cholinergic system by reducing the threshold of the presso-receptors in the gut wall. Possibly, a ganglionic blocking effect of serotonin in our intact preparation may have prevented its potentiation of acetylcholine at the neuroeffector site (28).

SUMMARY

1. An *in vivo* intra-arterial injection technique for the study of the effect of drugs upon the smooth muscle of the dog colon is described. Using this technique, the threshold dosage for acetylcholine and methacholine has ranged from 0.001 to 0.1 μg, and 0.001 to 1.0 μg., respectively.

2. Utilizing di-isopropylfluorophosphate, acute reduction of bowel cholinesterase (43 to 97 per cent) was achieved. This alteration was associated uniformly with augmentation of response to both acetylcholine and methacholine; however, at least 80 per cent inhibition was necessary to alter the threshold dose.

3. The action of 5-hydroxytryptamine (serotonin) and acetylcholine upon the colon of intact, anesthetized dogs was compared. The average threshold dose to induce contraction was the same for each substance.

4. Atropine was ineffective in blocking serotonin response as compared with acetylcholine, and inhibition of tissue cholinesterase failed to potentiate serotonin effect.

5. Although hexamethonium blocked neither acetylcholine nor serotonin response, mediation of action of these substances via autonomic ganglia is not excluded, since hexamethonium may act at the presynaptic level.

6. Effect of serotonin was enhanced by inhibition of monoamine oxidase and was blocked by both its benzyl analog and a phenothiazine derivative, prochlorperazine.

7. These results suggest that when serotonin is administered intra-arterially to an intact colon, it does not act via a cholinergic pathway; however, they do not negate previous evidence that this substance may be a modulator of the cholinergic system.

ACKNOWLEDGMENT

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THE EFFECT OF INSULIN ON NONESTERIFIED FATTY ACID RELEASE FROM THE HUMAN LEG*

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In recent years much interest has been focused on the plasma level of nonesterified fatty acid (NEFA), or unesterified fatty acid (UFA), and on its role as an energy substrate, particularly for muscle and liver.

Both insulin and glucose produce a rapid fall in serum NEFA level (1, 2), which is related to a decreased output of NEFA from the adipose cell (3, 4). The concept has arisen that in the fasting state NEFA is continuously mobilized from fat stores, and when glucose becomes available, this mobilization ceases and the blood level falls (5).

Several years ago Bell and Burns (6) showed that the intra-arterial injection of small doses of insulin into the leg of a human subject caused a prompt widening of the arteriovenous (A-V) difference for glucose across the injected limb as compared to the opposite noninjected limb. This apparent fixation of the insulin in the injected limb and the resulting differential metabolic effect seemed to offer an opportunity to study further the effect of insulin on NEFA handling by the peripheral tissues.

METHODS

Studies have been carried out on six normal fasting human subjects. Indwelling Cournand needles were placed in one femoral artery and in both femoral veins under procaine anesthesia. Simultaneous control samples were drawn from each of these three sites, followed by a small dose of glucagon-free insulin (one-half to one unit) injected intra-arterially. Thus insulin was injected directly into one limb, reaching the other limb after at least one circuit and in smaller concentration.

Care was taken to insure simultaneous withdrawal of all samples. Immediately after withdrawal, 5 ml. of blood was transferred from each syringe into fluoride tubes for glucose determination and the remainder was transported under refrigeration to the laboratory for centrifugation and determination of NEFA level according to the method of Dole (1). The analysis was done as rapidly as possible after withdrawal. In addition to analyses for sugar and NEFA, serum cholesterol was determined by the method of Abell, Levy, Brodie and Kendall (7) and triglycerides were determined by the method of Van Handel and Zilversmit (8). Blood sugar was done by the method of Somogyi (9, 10).

The insulin used for the study was hyperglycemic factor-free crystalline zinc insulin freshly prepared in saline for each experiment. The time required for injection was less than 10 seconds.

Six subjects were studied, observations being made on six injected limbs and six control limbs. All studies were carried out on lower extremities except for one control limb (Subject P). In this instance the arm was used instead of the leg because of difficulty in obtaining blood from the femoral vein.

RESULTS

The results are listed in Tables I through V. The changes to be emphasized are as follows.

1. Arterial NEFA level

The arterial level of NEFA at the control period ranged from 339 to 915 μMoles per L., all wtihin the accepted normal range. The mean arterial level of NEFA was significantly decreased after insulin injection (Table I). Although the relaxation of the subjects after the placement of the needles cannot be ruled out as a cause of the drop in this study (11, 12), such a drop in arterial NEFA has not been seen in other subjects receiving arterial injections of materials not affecting NEFA release.

2. A-V NEFA difference

Table II shows, for each subject, the A-V NEFA differences, both before and after insulin,

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† Research Fellow, American Heart Association.

TABLE I

Arterial nonesterified fatty acid level (micromoles per liter): Change after insulin

Subject	Control period	10 min. after insulin	Change during 10 min.	20 min. after insulin	Change during entire 20 min.
N	703	676	−27	525	−178
P	876	769	−107	504	−372
E	339	275	−64	206	−133
L	354	306	−48	306	−48
McI	485	446	−39	451	−34
McD	915	775	−140	648	−267
Mean	612	541	−71	440	−172
"t" value			3.96		3.23
p			<0.02		<0.05

for both the injected and noninjected limbs. In the injected leg, the initially negative A-V NEFA difference was narrowed at 10 minutes and, in four cases, reversed at 20 minutes. Changes in the A-V differences from the control period were large and uniform enough to be statistically significant (p being less than 0.05 at 10 minutes and less than 0.01 at 20 minutes after insulin injection). The noninjected limb showed no significant change in A-V differences.

Table III provides two further evidences that, regarding NEFA, insulin acted chiefly in the injected limb. Twenty minutes after insulin, the A-V NEFA change was significantly [1] different in the two limbs. Further, during the first 10 minutes, the change in A-V difference from the control condition was also significantly [2] different.

3. *Glucose*

The A-V glucose difference changes were as described by Bell and Burns (6): a widening in the injected limb, a narrowing in the uninjected limb (Tables IV and V).

4. *Triglyceride and cholesterol*

There was no significant alteration in triglyceride or cholesterol levels during the course of the study.

DISCUSSION

The tremendous responsiveness of the peripheral tissues of the leg to small doses of insulin is well shown in these studies. They confirm the reports of Bell and Burns (6) that insulin injected in small quantity into the artery supplying a single limb has a major effect on glucose uptake in this limb as compared to the remainder of the body; they show in addition that these effects extend to the NEFA as well as to glucose. It is felt that these studies show a primary effect of insulin on peripheral tissues, presumably adipose tissues, in regulating the level of NEFA as well as in regulation of blood glucose. The exit of NEFA from adipose tissues of the injected limb is reduced in contrast to the exit of NEFA from the uninjected limb.

It should be noted that at 20 minutes after in-

[1] In testing significance, the A-V difference across the control limb was subtracted for each subject from that for the injected limb, and the resulting number (*i.e.*, a difference of a difference) was tested against the null hypothesis.

[2] The change in A-V difference from the control condition was similarly compared between the two limbs. In effect, the noninjected limb was used as an individual control.

TABLE II

Arteriovenous (A-V) nonesterified fatty acid difference (micromoles per liter): Individual values

Subject	Control period		10 min. after insulin		20 min. after insulin	
	Injected limb	Noninjected limb	Injected limb	Noninjected limb	Injected limb	Noninjected limb
N	−163	−190	−69	−186	+44	−90
P	−71	+48*	+15	−77*	+57	−52*
E	−73	−73	+25	−51	−17	−42
L	−64	−84	−53	−119	+21	−60
McI	−40	−21	−46	−25	−21	−2
McD	−231	+3	−65	−15	+100	−58
Mean	−107.00	−52.83	−32.17	−78.83	+30.67	−50.67

* Brachial vein.

TABLE III

Arteriovenous (A-V) nonesterified fatty acid difference (micromoles per liter): Comparison of change in injected and noninjected limbs

	Control period	10 min. after insulin		20 min. after insulin	
	A-V difference	A-V difference	Change from control level	A-V difference	Change from control level
Injected limb	−107.0	−32.17	+74.83	+30.67	+137.67
Noninjected limb	−52.82	−78.83	−26.01	−50.67	+2.15
Difference	−54.18	+46.66	+100.84	+81.33	+135.52
"t" value	1.75	1.50	3.02	2.62	2.22
	(Not significant)	(Not significant)	($p<0.05$)	($p<0.05$)	(Not significant)

jection of insulin, the mean arterial level of NEFA usually exceeded the mean venous level of NEFA in the injected limb. This is not felt to represent an increased uptake of NEFA but a suppressed release of it which allowed the utilization of NEFA to exert a predominant effect on the venous level.

SUMMARY

1. The tissues of the leg exhibited extreme responsiveness to injection of small doses of insulin.

2. Arterial nonesterified fatty acid (NEFA) level dropped significantly within 20 minutes after intra-arterial injection of small doses of insulin.

3. The arteriovenous (A-V) NEFA difference in the injected limb changed significantly after insulin injection, from a negative A-V difference to a frequently positive A-V difference.

4. The A-V NEFA difference in the control limb did not change after insulin injection.

5. At 20 minutes after insulin injection the change in A-V NEFA difference in the injected limb was significantly different from that of the control limb and demonstrates a distinct action in the injected limb apart from the rest of the body.

6. The observed changes are considered to reflect a local suppression by insulin of NEFA release. This effect accompanies a local increase in glucose uptake, as was described by Bell and Burns (6).

TABLE IV

Arterial glucose (milligrams per 100 milliliters): Change after insulin

Subject	Control period	10 min. after insulin	20 min. after insulin
N	84.2	78.0	74.0
P	77.8	74.0	72.2
E	73.7	59.8	49.1
L	82.2	82.8	76.8
McI	96.0	83.9	75.1
McD	96.2	86.8	75.4
Mean	85.0	77.7	70.4

Mean change from control to 10 min. after insulin = (−)7.30 ("t" = 3.213, $p<0.05$).

Mean change from 10 to 20 min. after insulin = (−)7.28 ("t" = 5.009, $p<0.01$).

TABLE V

Glucose arteriovenous (A-V) difference (milligrams per 100 milliliters): Individual values

	Control period		10 min. after insulin		20 min. after insulin	
Subject	Injected limb	Noninjected limb	Injected limb	Noninjected limb	Injected limb	Noninjected limb
N	+3.4	+4.2	+4.2	+1.4	+7.0	+2.3
P	+4.9	+3.8*	+7.5	+0.2*	+12.9	+3.1*
E	+0.7	+4.3	+10.9	−0.5	+9.2	+2.3
L	+2.1	+0.7	+2.8	+3.1	+13.6	+1.0
McI	0.0	+0.6	+5.0	−1.0	+7.9	−2.4
McD	+4.4	+3.6	+13.1	−0.9	+14.6	−0.5
Mean	+2.58	+2.87	+7.25	+0.38	+10.87	+0.97
Mean difference	−0.31		6.87		9.90	
	"t" = 0.41 (Not significant)		("t" = 3.19, $p<0.05$)		("t" = 8.52, $p<0.01$)	

* Brachial vein.

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ELECTROPHORETIC STUDIES OF THE VITAMIN B_{12}–BINDING PROTEIN OF NORMAL AND CHRONIC MYELOGENOUS LEUKEMIA SERUM *

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The serum in chronic myelogenous leukemia (CML) has an increased concentration of vitamin B_{12} (1, 2) and an increased capacity to bind added vitamin B_{12} either *in vivo* (3–5) or *in vitro* (2, 6, 7). The vitamin B_{12} of normal and CML serum moves with the α-globulins when serum is electrophoresed at pH 8.6 (1, 8, 9). The seromucoid, the fraction of serum containing glycoproteins which comprises about 1.5 per cent of the serum proteins (10), moves with the α-globulins when electrophoresed at pH 8.6 (11). Furthermore, it has been found that the seromucoid fraction isolated from CML serum is capable of binding amounts of added cobalt^{60}-labeled vitamin B_{12} (B_{12}*) sufficient to account for the increased binding capacity of the whole serum (12). The possibility that the binding protein of both normal and CML serum for native vitamin B_{12} (B_{12}BP) is in the seromucoid fraction was investigated.

METHODS

Venous blood was collected in the fasting state from normal subjects and from patients with CML in hematological and clinical relapse. About one to two hours after clotting, the serum was separated and either used immediately or stored at -20°C. The seromucoid fraction of serum was prepared by the method of Winzler, Devor, Mehl and Smyth (10) as follows: one volume of serum was added to one volume of 0.4 M sulfosalicylic acid, thoroughly mixed for five minutes, and then centrifuged. The supernatant containing the seromucoid was separated and neutralized with 1 N NaOH. The neutralized seromucoid and the original serum from which it had been prepared were dialyzed against large volumes of 0.15 M NaCl for 72 hours at 4 to 6°C. and its vitamin B_{12} concentration then determined by *Euglena gracilis* assay (13). When used for electrophoresis, the seromucoid was dialyzed for 72 hours against large volumes of distilled water, lyophilized, and the dried protein then dissolved in the appropriate buffer. Seromucoid or serum containing bound (nondialyzable) B_{12}* [1] was prepared by adding an amount of B_{12}* in excess of the binding capacity (4 mμg. B_{12}* per ml. of normal serum or its equivalent seromucoid and 12 mμg. per ml. of CML serum or its equivalent seromucoid). The seromucoid was then dialyzed against large volumes of distilled water for 72 hours at 4 to 6° C. and lyophilized, while the serum was dialyzed against 0.15 M NaCl.

Lyophilized Cohn plasma fractions (I through VI),[2] prepared by Method VI (14), were dissolved in sufficient 0.15 M $NaHCO_3$ to bring them to their equivalent plasma volumes, dialyzed for 48 hours against 0.15 M NaCl, and the vitamin B_{12} concentration determined as was the vitamin B_{12} concentration of the original plasma.

Zone electrophoretic separation of serum or lyophilized seromucoid was performed using starch blocks as the supporting medium. The general technique described by Kunkel (15) was used except that the electrophoresis was carried out at pH 4.5 which permitted separation of the B_{12}BP from the bulk of serum proteins. The starch blocks were prepared from slurries of starch [3] that had been previously washed once with distilled water and twice with an acetate-sodium chloride buffer pH 4.5, ionic strength 0.10. The buffer consisted of 0.04 M sodium acetate and 0.06 M NaCl with concentrated HCl added to bring the pH to 4.5. The following samples were electrophoresed: *a*) a mixture of 1.0 ml. of serum and 0.5 ml. of acetate buffer; *b*) serum containing bound (nondialyzable) B_{12}* in a volume of 1.5 to 2 ml.; *c*) lyophilized seromucoid dissolved in a solution of 1 ml. of 0.15 M NaCl and 0.5 ml. acetate buffer; and *d*) lyophilized seromucoid containing bound B_{12}* dissolved as in *c*. Samples were applied to a 4 to 5 mm. transverse slit at the center of the block. In some experiments, serum containing bound B_{12}* was run in parallel on the same starch block as the native serum. Electrophoresis was carried out for 16 hours at 5 to 8° C. using a current flow of 36 ma. for a 31 cm. long, 10 cm. wide, 1.5 cm. thick starch block. At the termination of electrophoresis a maximum

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[1] Kindly supplied by Dr. N. S. Ritter, Merck and Co., Rahway, N. J.

[2] Kindly prepared by the Plasma Protein Foundation, Boston, Mass.

[3] Fisher hydrolyzed potato starch, Fisher Scientific Co., New York, N. Y.

TABLE I

The native vitamin B_{12} concentration of the seromucoid of normal and chronic myelogenous leukemic (CML) sera

Subject		Serum B_{12} concentration	Seromucoid B_{12} concentration	Seromucoid fraction
		$\mu\mu g./ml.$	$\mu\mu g./ml.$	%
Normal				
1	S.W.	525	435	83
2	L.S.	125	122	98
3	A.N.	732	451	62
4	M.B.	732	539	74
5	T.C.	681	544	80
6	S.J.	332	208	63
Mean		521	383	77
Range		125–732	123–544	62–98
CML				
1	H.W.	6,096	5,976	98
2	A.M.	15,121	15,101	100
3	C.M.	9,220	9,010	98

pH change of 0.2 pH unit was found at either end of the gel. After drying at room temperature (0.5 to one hour) the gel was cut into transverse segments so that the origin was included in one segment (O segment). Other segments were designated as anodal (A) or cathodal (C) and identified by the number of segments from the O segment. Sections of the block at the cathodal and anodal ends well outside the range of migration of the proteins were removed to serve as blanks. Each segment was suspended in 8 ml. of distilled water, the contents periodically mixed for 1.5 hours, and then centrifuged. The supernatant was removed and centrifuged again. The vitamin B_{12} concentration of these eluates was then determined by *E. gracilis* assay. The per cent recovery of vitamin B_{12} from the starch block in 19 electrophoretic analyses of serum or seromucoid was equal to an average of 93 per cent ± 14, range, 75 to 115 per cent.

The protein concentration of starch eluates was determined by Kunkel and Tiselius' modification (16) of the Folin-Ciocalteu procedure (17). Protein concentration was plotted in terms of optical density with necessary corrections made for the volume of eluate analyzed. Sialic acid was determined by the method of Werner and Odin (18) using 2 ml. of the eluates. Because of the negligible reading of the blank with Baker's iso-amyl alcohol, further purification of the iso-amyl alcohol was not necessary.

In preliminary experiments, it was found that albumin and the bulk of the globulins remained at the origin or migrated cathodally when serum was electrophoresed at pH 4.5. Acidic α_1-glycoprotein,[4] isolated from Cohn Fraction VI (19), was dissolved in a solution composed of 1 ml. 0.15 M NaCl and 0.5 ml. acetate buffer, electrophoresed on a starch block, (pH 4.5) and the protein and sialic acid concentration of 1.0 cm. segments of the block determined.

After electrophoresis, the starch segments containing nondialyzable B_{12}* were placed in test tubes and the radioactivity determined in a plastic scintillation well counter (20) having a background of 385 to 400 counts per minute. Samples with higher counting rates, such as *a*) the origin and first four cathodal segments of normal sera, *b*) anodal segments of the seromucoid and *c*) the anodal and cathodal segments of CML sera, were counted long enough to give a 10 to 15 per cent counting error. The anodal segments of the normal sera contained small amounts of radioactivity (as little as five to 10 counts per minute over background) and were counted for three to four 10 minute periods with 10 minute counts of background alternating between each sample count. The radioactivity of the B_{12}* solution added to serum was counted in the same well counter using similar geometry, and the concentration of vitamin B_{12} in this solution was also determined. From the *a*) specific activity of the added B_{12}* and *b*) radioactivity found in each starch segment, the amount of B_{12}* ($\mu\mu g.$) per segment was estimated. The total vitamin B_{12} bound by the proteins in any starch segment was calculated by summing of the native vitamin B_{12} (determined by *E. gracilis* assay) and of the nondialyzable B_{12}*.

RESULTS

Vitamin B_{12} concentration of the seromucoid

The seromucoid contained an average of 77 per cent, range 62 to 98 per cent, of the vitamin B_{12} of whole normal serum (Table I). Despite a great increase in serum vitamin B_{12} concentration, 98 to 100 per cent of the vitamin was recovered from the seromucoid in three patients with CML.

Vitamin B_{12} concentration of Cohn plasma fractions

Vitamin B_{12} was found in all six Cohn plasma fractions. Fraction V contained the highest percentage of the plasma vitamin (27 per cent) while Fraction VI had only 14 per cent.

Vitamin B_{12} concentration of fractions of the seromucoid separated by electrophoresis

The distribution of native vitamin B_{12}, protein and sialic acid in the seromucoid, obtained from normal serum following starch gel electrophoresis at pH 4.5, is shown in Figure 1. The protein and sialic acid were concentrated in segments anodal to the origin with peak levels found in the A-3 and A-4 segments. A similar distribution of protein and sialic acid was found when a crystalline preparation of the acidic α_1-glycoprotein was electro-

[4] Kindly supplied by Dr. Karl Schmid, Massachusetts General Hospital, Boston, Mass.

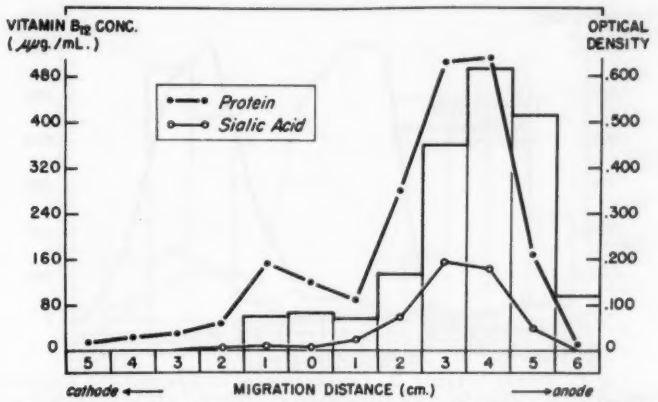

FIG. 1. NATIVE VITAMIN B_{12} CONCENTRATION OF FRACTIONS OF NORMAL SEROMUCOID SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT PH 4.5

Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Optical density is plotted on the right ordinate with protein the solid dots ●——●, and sialic acid the open dots ○——○.

phoresed at pH 4.5. The vitamin B_{12} was also found anodally with peak concentrations at A-3 to A-5 segments. The B_{12}* bound to the normal seromucoid had a distribution similar to the native vitamin, with peak concentrations at A-2 to A-4 segments (Figure 2).

The distribution of protein, sialic acid and vitamin B_{12} in the CML seromucoid was similar to that of the normal, with peak concentrations found at A-3 to A-4 segments (Figure 3). Furthermore, the distribution of added B_{12}* bound by CML seromucoid was similar to that of the native vitamin, with peak concentrations located at A-2 to A-4 segments (Figure 4).

Vitamin B_{12} concentration of fractions of serum separated by electrophoresis

Electrophoresis of a normal serum at pH 4.5 (Figure 5) is representative of the findings in a group of eight such sera. In sharp contrast to the seromucoid, there was a negligible amount of protein in A-2 to A-6 segments with the peak pro-

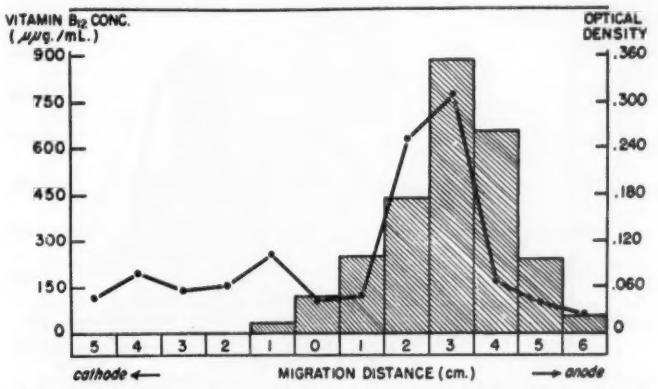

FIG. 2. DISTRIBUTION OF BOUND (NONDIALYZABLE) B_{12}* AMONG FRACTIONS OF NORMAL SEROMUCOID SEPARATED BY STARCH GEL ELECTROPHORESIS AT PH 4.5

The concentration of B_{12}* is plotted on the left ordinate and is represented by the ruled boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

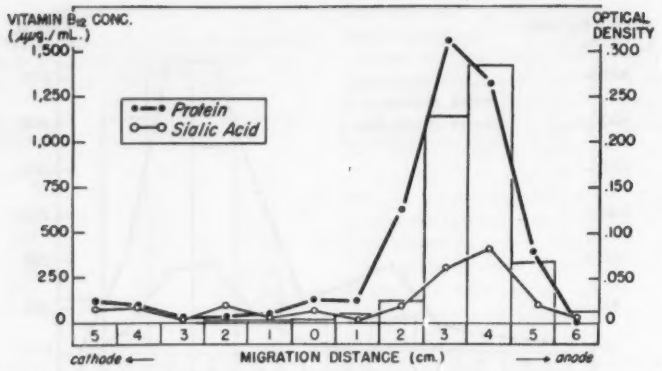

FIG. 3. NATIVE VITAMIN B_{12} CONCENTRATION OF FRACTIONS OF CHRONIC MYELOGENOUS LEUKEMIA SEROMUCOID SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT pH 4.5

Vitamin B_{12} concentration plotted on the left ordinate is represented by the open boxes. Optical density is plotted on the right ordinate with protein the solid dots ●——● and sialic acid the open dots ○——○.

tein concentration at the O segment (A-1 or C-1 in some sera). However, virtually all of the native vitamin B_{12} was recovered anodally (83 to 92 per cent found in segments A-2 to A-6), with peak concentrations in A-3 to A-5 segments. The distribution of the native vitamin B_{12} and of the nondialyzable B_{12}* bound to the same serum is shown in Figure 6 and is representative of the findings in a group of seven such sera. The native vitamin had the same anodal distribution as previously described. Only a small amount of B_{12}* was found anodally, segments A-2 to A-6 containing an average of 80 μμg. per ml. ± 17, range, 28 to 183 μμg. per ml. The proteins with the greatest binding ability for the added B_{12}* were found at the origin and C-1 to C-3 segments. The binding of B_{12}* by A-2 to A-6 segments was equal to an average of 6 per cent ± 4, range, 3 to 15 per cent of the total bound radiovitamin. The saturation of the B_{12}BP (located at A-2 to A-6 segments) as estimated from the ratio of the native bound vitamin to the total vitamin that could be

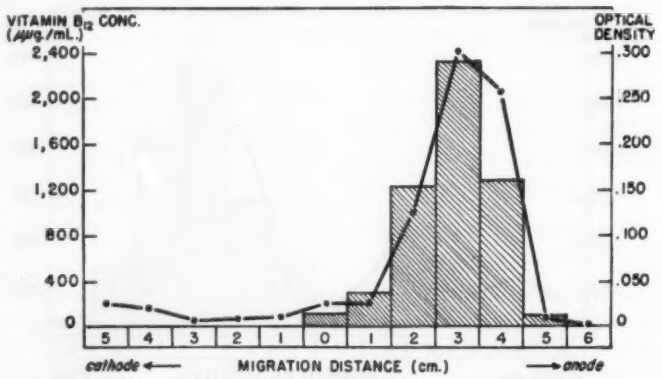

FIG. 4. DISTRIBUTION OF BOUND (NONDIALYZABLE) B_{12}* AMONG FRACTIONS OF CHRONIC MYELOGENOUS LEUKEMIA SEROMUCOID SEPARATED BY STARCH GEL ELECTROPHORESIS AT pH 4.5

The concentration of B_{12}* plotted on the left ordinate is represented by the ruled boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

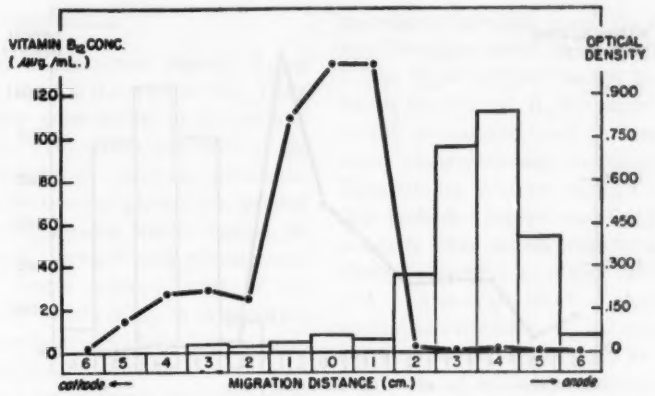

FIG. 5. NATIVE VITAMIN B_{12} CONCENTRATION OF PROTEIN FRACTIONS OF NORMAL SERUM SEPARATED BY STARCH GEL ELECTROPHORESIS AT PH 4.5

Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

bound and was equal to an average of 78 per cent ± 13, range, 57 to 94 per cent.

The distribution of vitamin B_{12} in a CML serum (Figure 7) is representative of a group of four such sera. As in the normal, the protein concentration of the A-2 to A-6 segments was negligible, while the native vitamin B_{12} was virtually all found anodally with peak concentrations at A-3 to A-5 segments. The distribution of the native vitamin and of the nondialyzable B_{12}* (Figure 8) is representative of a group of three such sera. The native vitamin had the same anodal

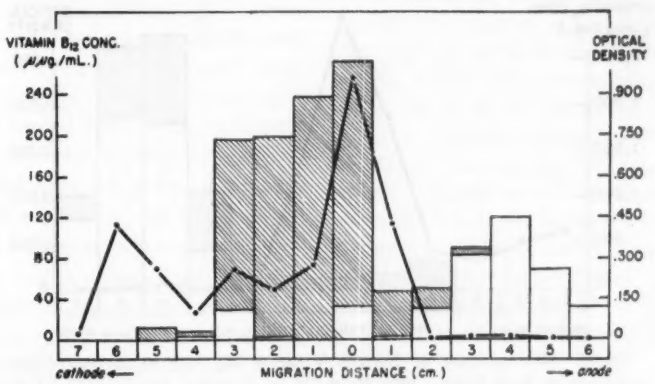

FIG. 6. DISTRIBUTION OF NATIVE VITAMIN B_{12} AND OF BOUND (NONDIALYZABLE) B_{12}* AMONG THE PROTEINS OF NORMAL SERUM SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT PH 4.5

Normal serum and an aliquot of the same serum containing bound (nondialyzable) B_{12}* were electrophoresed on the same starch block. The native vitamin shown by the open boxes was measured by *Euglena gracilis* assay while the bound radiovitamin, shown by the ruled boxes, was measured by counting the radioactivity in each segment. The solid dots ●——● refer to protein concentration.

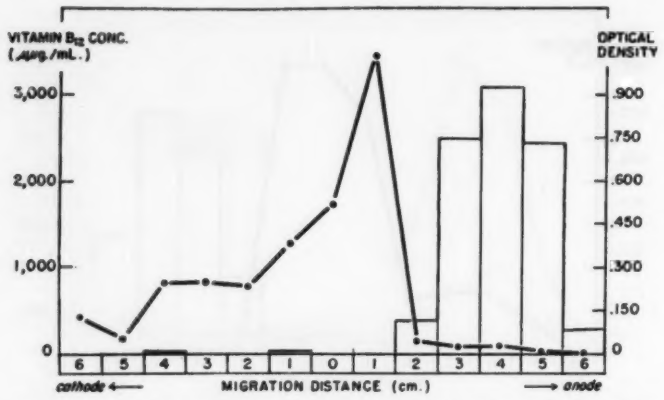

FIG. 7. NATIVE VITAMIN B_{12} CONCENTRATION OF PROTEIN FRACTIONS OF CHRONIC MYELOGENOUS LEUKEMIC SERUM SEPARATED BY STARCH GEL ELECTROPHORESIS AT pH 4.5

Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots ●——●.

distribution as described above. However, in contrast to the normal, a large amount of the added B_{12}* (2,200 to 3,973 μμg. per ml.) was bound by the A-2 to A-6 segments. The binding of the radiovitamin by these anodal segments was equal to 48 to 78 per cent of the total serum binding of B_{12}*. The per cent saturation of the B_{12}BP located at A-2 to A-6 segments ranged from 35 to 75 per cent, as calculated by the method outlined above.

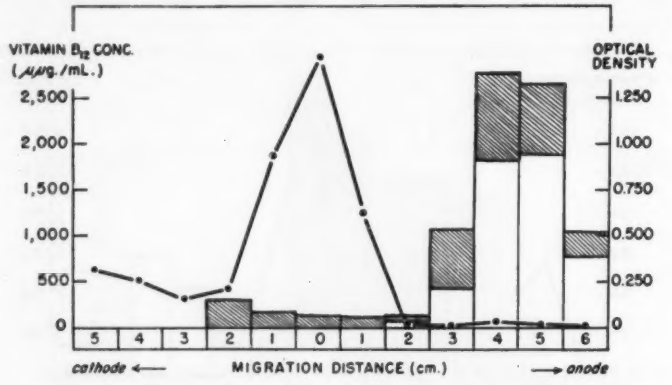

FIG. 8. DISTRIBUTION OF NATIVE VITAMIN B_{12} AND OF BOUND (NONDIALYZABLE) B_{12}* AMONG THE PROTEINS OF CHRONIC MYELOGENOUS LEUKEMIC (CML) SERUM SEPARATED BY ELECTROPHORESIS ON STARCH GEL AT pH 4.5

CML serum and an aliquot of the same serum containing bound (nondialyzable) B_{12}* were electrophoresed on the same starch block. The native vitamin shown by the open boxes was measured by *Euglena gracilis* assay while the bound radiovitamin shown by the ruled boxes was measured by counting the radioactivity in each segment. Note the change in scale of vitamin B_{12} concentration plotted on the left ordinate from that of Figure 6. The solid dots ●——● refer to protein concentration.

DISCUSSION

A large fraction of the native vitamin B_{12} of normal serum was found in the seromucoid. Cohn Fraction VI, thought to be similar to the seromucoid in composition (19), contained little of the vitamin. In Method VI of Cohn and associates, the major protein fractions of plasma are isolated by a series of precipitations which depend on changes in pH, ionic strength and ethanol concentration (14). Some coprecipitation of the seromucoid routinely occurs when it is prepared from serum by precipitation of the serum proteins with sulfosalicylic or perchloric acid (10), and similarly it may be presumed that coprecipitation of the B_{12}BP with each Cohn plasma fraction may account for the absence of selective concentration of the vitamin in Fraction VI.

Since the great bulk of serum proteins have isoelectric points of 4.5 or higher, they either remained at the origin or migrated cathodally at this pH. However, due to the more acidic isoelectric point of the B_{12}BP, it migrated anodally with the other proteins of the seromucoid. The native vitamin B_{12} in serum or seromucoid preparations was usually distributed over a relatively wide anodal area of the starch block, with peak concentrations found in a 2 to 3 cm. width. Trailing of the protein and/or diffusion of the B_{12}BP during the electrophoretic run may account for the wide distribution of the vitamin and does not necessarily indicate the presence of more than one binding protein. The anodal distribution of vitamin B_{12} was simliar to, but not identical with, that of the main protein and sialic acid peaks of the seromucoid or its chief constituent, the acidic α_1-glycoprotein (19, 21). Despite their somewhat similar mobility, the B_{12}BP is probably not the acidic α_1-glycoprotein, since *1*) the B_{12}BP was not selectively precipitated with Cohn Fraction VI, whereas the acidic α_1-glycoprotein has been found only in this fraction (19); and *2*) Fahey, McCoy and Goulian fractionated serum on a diethylaminoethyl cellulose ion-exchange column and found that the B_{12}BP was eluted off before the acidic α_1-glycoprotein (22). A small amount of the native vitamin B_{12} of normal serum (10 per cent) was found scattered among cathodally moving proteins. It is impossible to know whether this represents technical error in the measuring of small amounts of the vitamin or another B_{12}BP.

The B_{12}BP of CML serum had properties similar to the normal B_{12}BP since *1*) it was found in the seromucoid, and *2*) had anodal mobility when electrophoresed on starch gel at pH 4.5. Mendelsohn, Watkin, Horbett and Fahey found that both the normal and CML B_{12}BP behaved similarly when serum was chromatographed on a diethylaminoethyl cellulose ion-exchange column (23). These chemical similarities between the normal and the CML B_{12}BP strongly suggest that the increased concentrations of vitamin B_{12} uniformly found in CML serum in relapse is due to an increase in concentration of the normal B_{12}BP rather than to an abnormal protein. Despite the tremendous increases in concentration of the B_{12}BP in CML sera (amounting to as much as 100-fold in one patient we have studied), only slight increases in the concentration of the total seromucoid fraction have been reported in this disease (24). However, the concentration of B_{12}BP in normal serum is probably minute as compared to that of the total seromucoid. Thus, the B_{12}BP of a typical normal serum, when completely saturated, can bind approximately 0.65 mμg. per ml. If the molecular weight of the B_{12}BP is similar to that of the orosomucoid, *i.e.*, 44,000 (25), and if one molecule of the B_{12}BP can bind one molecule of vitamin B_{12} (molecular weight, 1,343), the concentration of normal B_{12}BP would be equal to 2.20 μg. per 100 ml. serum. Even a 100-fold increase in concentration of B_{12}BP, to 0.22 mg. per 100 ml., would represent only an increase of 0.2 per cent over the normal concentration of the seromucoid, an increase well within the error of the chemical determination. It would appear that the elevations in seromucoid concentration found in CML are contributed by constituents of the seromucoid other than the B_{12}BP.

When additional B_{12}* was added to normal serum, the bound (nondialyzable) radiovitamin was almost all associated with proteins found at the origin or cathodal segments, whereas a very small amount of the added B_{12}* was bound by the anodally migrating B_{12}BP. This suggests that at the levels of vitamin B_{12} found in normal serum, the B_{12}BP approaches saturation and, therefore, the addition of further vitamin results

in the binding of the vitamin to nonacidic proteins. In CML serum on the other hand, there appears to be a commensurate increase of the B_{12}BP in association with the increased level of vitamin B_{12}, so that although the relative saturation of the former remains approximately normal, the absolute binding capacity for added vitamin B_{12} is greatly increased.

The B_{12}BP has chemical properties similar to those of the acidic α_1-glycoprotein and the α_2-glycoprotein isolated from Cohn Fraction VI (19, 26). Thus, they are all relatively heat resistant, are all soluble in sulfosalicylic or perchloric acid, are all precipitated by phosphotungstate in 2 N HCl and all have acidic isoelectric points as compared with other serum proteins (11, 12, 19, 21, 26, 27). The acidic α_1-glycoprotein (19) and the α_2-glycoprotein (26) are mucoid glycoproteins, *i.e.*, proteins containing significant amounts of firmly bound hexosamine (28). This would suggest that the B_{12}BP may be a mucoid glycoprotein, although direct confirmation of such a structure must await its isolation in pure form. It is of interest that intrinsic factor (29, 30) and erythropoietin (31) are probably also mucoid glycoproteins. The available evidence suggests that the B_{12}BP is not, however, identical with either of these substances. Thus, intrinsic factor has a differing electrophoretic mobility at pH 8.6 (32) and its binding sites for vitamin B_{12} are relatively heat labile (33). Recently, we have found a vitamin B_{12}-binding substance in CML urine which resembles that found in CML serum (34). This urinary material, when given orally to patients with pernicious anemia in amounts sufficient to bind an oral dose of B_{12}*, had no intrinsic factor activity. Also, increased erythropoietin levels have been reported in such diseases as chronic lymphatic leukemia (35), carcinoma of the cervix (35), secondary polycythemia (36) and hypoplastic anemias (37), whereas vitamin B_{12} levels are not particularly increased in these conditions.

It seems reasonable to suppose that the B_{12}BP functions as a transport protein for vitamin B_{12}. The seromucoid fraction, representing a readily accessible, easily prepared, and easily concentrated source of the B_{12}BP, should aid in experiments designed to elucidate the role played by this protein in vitamin B_{12} metabolism.

SUMMARY

1. A method for the separation of the vitamin B_{12}-binding protein (B_{12}BP) of normal and chronic myelogenous leukemia serum by electrophoresis on starch gel at pH 4.5 has been described.

2. The B_{12}BP of normal serum has been identified as a constituent of the seromucoid fraction of serum. It is a protein with an electrophoretic mobility at pH 4.5 similar to that of the acidic α_1-glycoprotein, the most acidic protein found in serum. However, it was not selectively precipitated with Cohn Fraction VI.

3. The B_{12}BP of chronic myelogenous leukemia serum had properties similar to the normal B_{12}BP, suggesting that the great increase in vitamin B_{12} levels found in chronic myelogenous leukemia sera results from an increased concentration of the normal B_{12}BP rather than from an abnormal protein.

4. Only a very small fraction of the cobalt^{60}-labeled vitamin B_{12} bound by normal serum as determined by dialysis was bound to the B_{12}BP. In contrast to the normal, the largest fraction of the cobalt^{60}-labeled vitamin B_{12} bound by chronic myelogenous leukemia serum was bound by the B_{12}BP.

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THE IDIOPATHIC HIGH CARDIAC OUTPUT STATE *

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The high cardiac output state has occasioned much interest in recent years, particularly when associated with clinical heart failure. The etiology of a high cardiac output is often clinically obvious or easily ascertained by pertinent laboratory tests. Most persistent elevations of cardiac output can be attributed to arteriovenous fistulae, to arterial anoxemia or anemia, or to metabolic or hormonal alterations in vasomotor tone and blood flow distribution. Isolated elevations of blood flow unexplained by these mechanisms can usually be related to the occurrence of anxiety at the time of study (1).

Although a discrete alteration in the "set" of the regulatory mechanism for cardiac output has yet to be described, disturbances of the mechanism for the regulation of heart rate (2) and blood pressure (3) are well known. On the other hand, almost every cardiovascular laboratory has seen an occasional patient in whom cardiac output is persistently and inexplicably increased. It is the purpose of this report to present a group of individuals who show in association a persistently elevated cardiac output, cardiac murmur and cardiac hypertrophy. No specific etiology for these changes could be demonstrated by known clinical or laboratory methods. The patients came to our attention initially either because of the presence of unexplained cardiac murmurs detected on routine physical examination [1] or because of abnormal electrocardiograms.

MATERIALS AND METHODS

Eight patients were studied. A complete history was obtained and physical examination performed. A 12-lead electrocardiogram, urinalysis and chest X-ray and fluoroscopy were obtained; and the hematocrit and blood urea nitrogen determined. Cardiac catheterization was carried out in all with measurement of pulmonary and systemic pressures. Left to right intracardiac shunts were sought by analysis of oxygen or nitrous oxide samples (4) drawn consecutively from the pulmonary artery, right ventricle, right atrium, venae cavae and innominate vein. Cardiac output was measured during the catheterization by the direct Fick method. Repeat outputs were obtained on at least one and usually two or more subsequent occasions, utilizing both continuous sampling and external surface counting direct writing isotope (I^{131} albumin) dilution techniques (5).

A total of 30 measurements of cardiac output was made in eight individuals during the resting state. Values were recorded during a four minute exercise period in six patients and 10 minutes after *recovery* from effort, at which time pulse rate was at or below pre-exercise levels. Outputs were estimated one to two hours following sedation in five patients. In addition, in five patients cardiac outputs were determined only after nighttime sedation had been followed by a four to five hour period of rest (or sleep) in a darkened room free of auditory and visual stimuli. The brachial arterial pressure curve was recorded on at least two occasions with measurements of phasic and mean pressures and the duration of systolic ejection. Arterial pH, pCO_2 and O_2 saturations were determined (6); blood lactates and pyruvates were analyzed [2] (7, 8); thyroid function was studied by means of basal metabolic rate, 24 hour I^{131} uptake and measurement of protein-bound iodine. Blood catechol amines [2] were measured by the Aronow and Howard modification of the method of Weil-Malherbe and Bone (9, 10) at rest and on exercise. Calculations of cardiac work and peripheral vascular resistance were carried out as described elsewhere (4).

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[2] The authors are grateful to Drs. Dale Friend and Albert Renold of the Harvard Medical School through whose laboratory assistance we were able to obtain catechol amine levels and lactate-pyruvate studies.

TABLE I

Clinical, ECG and X-ray findings in patients with idiopathic high output state

	G. S.	R. L.	L. P.	R. A.	R. R.	B. H.	T. M.	A. N.	Total
Clinical examination									
Related symptoms	0	0	0	0	0	+	0	+	2/8
Systolic murmur:									
Duration	1 yr.	19 yrs.	6 wks.	15 yrs.	2 yrs.	42 yrs.	24 yrs.	10 yrs.	
Location and radiation	LSB* to base	3 LIS† to rt. P-C‡	LSB to apex + base	base	LSB to apex	apex to P-C	3 LIS to base + P-C	LSB to base + apex	
Characteristics	short mid-systolic	blowing pan-systolic	blowing early systolic	blowing pan-systolic	short mid-systolic	blowing pan-systolic	short high-pitched systolic	rough pan-systolic	
Grade	II	III	II	III	II	IV	IV	III	
Overactivity:									
Carotid artery	+	N	+	+	+	+	+	+	7/8
Left ventricle	N	+	+	+	N	+	+	+	6/8
Right ventricle	N	N	N	N	N	+	N	+	2/8
Systolic hypertension	+	+			+			+	4/8
ECG									
Right ventricular hypertropy						+			1/8
Incomplete right bundle branch block		+					+	+	3/8
Left ventricular hypertrophy	+		+	+	+		+	+	6/8
X-ray									
Cardiac enlargement						+		+	2/8
Right ventricular prominence						+	+	+	3/8
Left ventricular prominence					+		+	+	3/8
Pulmonary artery prominence					+	+	+	+	4/8
Pulmonary vascular prominence	+				+	+	+	+	5/8

* LSB = left sternal border.
† LIS = third left intercostal space.
‡ P-C = precordium.

Subjects

All were males between the ages of 17 and 48 with an average age of 27 years. One patient had associated mild mitral stenosis, atrial fibrillation and disabling pulmonary symptoms. Of the remaining seven patients, only one (A. N.) had symptoms referable to the heart. He had moderate exertional dyspnea and fatigue and was the patient with the greatest cardiac enlargement; he also showed electrocardiographic evidence of combined ventricular involvement. All eight patients could be characterized as "anxious" but, nevertheless, effective individuals. One was a trained athlete, and three were engaged actively in sports. Patients T. M., R. L. and B. H. have had known heart murmurs since childhood.

The patients as a group were well-developed, well-nourished individuals with normal eating habits and average diets and, excluding the patient with mitral stenosis, revealed the following physical findings. A precordial systolic murmur varying from Grade II to IV [3] was heard in all patients; the murmurs varied in locale somewhat but were loudest along the left sternal border with frequent radiation to the base or apex; the quality and configuration of systolic murmur varied from patient to patient (Table I). Position and respiration had no consistent effect, while effort increased the intensity of the murmur. A right ventricular (left sternal border) heave was noted in two, a forceful apical thrust (left ventricular overactivity) in six, and hyperactivity of the carotid arteries in seven. Blood pressure was noted to be labile from examination to examination, and four of the individuals showed a moderate systolic hypertension. No bruits were noted over any of

[3] Graded on a scale I to VI.

TABLE II

Special laboratory tests in patients with high cardiac output

Patient	Hematocrit	Total blood volume	Protein-bound iodine	Systemic arterial			Plasma catechol amines		Lactate	Pyruvate	Cold pressor test
				pH	p CO_2	O_2 sat.	Epinephrine	Norepinephrine			
		L./M.²	*µg./100 ml.*		*mm. Hg*	%	*µ/L.*	*µ/L.*	*mg./100 ml.*	*mg./100 ml.*	
G. S.	48	2.5	4.0	7.37	34	94	5.7	1.2	8.6	0.48	+
R. L.	47	3.8	4.8	7.42		97	0.3	1.5	10.6	0.5	+
L. P.	50	2.7		7.36	40	96					
R. A.	45	3.3	5.4	7.40	34	96	0.9	3.2	8.2	0.23	+
R. R.	38	3.2	3.0	7.43	32	95	0	1.1	7.6	0.46	neg.
B. H.	45		24%*			92					
T. M.	50	2.7	5.0	7.39	42	97	1.0	2.2	12.3	0.39	+
A. N.	43	4.3	6.0	7.39	35	97	1.5	6.7	15.9	0.47	+
Average	46	3.2	4.6	7.39	36	95	1.2	2.4	10.5	0.42	5/6

* Twenty-four hour I^{131} uptake.

the limbs or visceral organs, and there was no evidence of skin or mucous membrane angiomas. Electrocardiographic examination (Table I) revealed right ventricular hypertrophy [4] in one; incomplete right bundle branch block [4] in three and left ventricular hypertrophy [4] in six. In Patient T. M. hypertrophy had been known to be present for 13 years. It is perhaps of greatest importance that in no patient was the electrocardiogram entirely within normal limits. X-ray examination (Table I) revealed a normal size heart in six of the eight patients. The patient with mitral stenosis (B. H.) showed enlargement in the typical mitral configuration. The eighth case (A. N.) revealed generalized cardiomegaly with enlargement of both right and left ventricles and marked pulmonary arterial plethora. Prominence of the right ventricle, as indicated by shouldering in the left anterior oblique view and contiguity of half of the cardiac silhouette with the anterior chest

[4] Right ventricular hypertrophy was indicated by right axis deviation, R/s quotient greater than one in V_1 and associated T wave changes. Incomplete right bundle branch block was evidenced by a polyphasic ventricular complex (type r s R′, RsR′, and so forth) with a duration of less than 0.12 second in right precordial leads. Left ventricular hypertrophy was inferred from increased voltage (QRS greater than 26 mm. in V_5 or V_6 or S_{V_1} plus $R_{V_5 \text{ or } 6}$ greater than 35 mm.) and delayed intrinsicoid deflection in left precordial leads (11). Three of the cases with left ventricular hypertrophy showed the slightly elevated, upwardly concave ST segments and peaked T waves in the left precordium that have been described as "diastolic overloading" (12).

TABLE III

Hemodynamic data at rest during cardiac catheterization in patients with high cardiac output

Patient	Age	Body surface area	Oxygen consumption	Arteriovenous oxygen difference	Cardiac index	Heart rate	Stroke index	Brachial artery			Systemic vascular resistance	Left ventricular work index
								Pressure		Systolic ejection period		
								S/D*	Mean			
		M.²	*cc./min./M.²*	*vol. per cent*	*L./min./M.²*	*min.*	*cc./beat/M.²*	*mm. Hg*	*mm. Hg*	*sec./beat*	*dynes-sec.-cm.⁻⁵*	*Kg. M./min./M.²*
G. S.	18	1.85	197	3.4	5.8	65	89	147/94	109	0.36	816	9.9
R. L.	22	1.78	205	3.0	6.8	95	72	150/75	95	0.24	625	10.8
L. P.	17	1.73	162	2.7	6.0	81	74	122/74	94	0.28	735	8.3
R. A.	35	1.80	158	2.6	6.1	77	79	105/65	85	0.33	675	7.7
R. R.	18	2.05	210	2.7	7.8	76	103	148/75	100	0.28	432	13.3
B. H.	48	1.67	171	2.7	6.3	105†	60	152/82	110	0.26	869	11.0
T. M.	27	1.73	125	2.2	5.7	76	75	124/55	78	0.28	650	8.0
A. N.	27	1.88	189	2.9	6.5	77	84	143/81	103	0.32	600	11.3
Average	27	1.81	177	2.8	6.4	81	80	136/75	95	0.29	657	10.0

* S/D = systolic/diastolic.
† Auricular fibrillation.

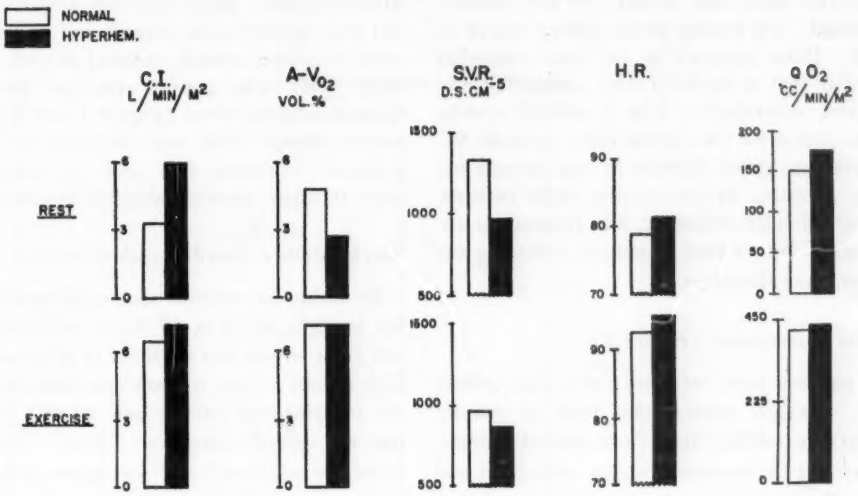

FIG. 1. COMPARISON OF AVERAGE HEMODYNAMIC VALUES AT REST AND DURING EFFORT BETWEEN NORMALS AND PATIENTS WITH HIGH CARDIAC OUTPUT

CI = cardiac index; A-V O_2 = arteriovenous oxygen difference; SVR = systemic vascular resistance (dynes-sec.-cm.$^{-5}$); HR = heart rate; qO_2 = oxygen consumption.

wall, was seen in three patients. Prominence of the left ventricle, as indicated by increased convexity of the left border in the posteroanterior view and posterior enlargement in the left anterior oblique view, was seen in three individuals. The main pulmonary artery was increased in four and the vasculature was prominent in five. X-ray abnormalities had been present in Patient T. M. for the past 15 years.

Clinical laboratory tests

Hematocrit reading (Table II), blood urea nitrogen and urinalysis in all patients were normal. Bone films taken in one individual, whose head appeared to be slightly enlarged, revealed no evidence of Paget's or other bone disease. A cold pressor test was positive in five out of six patients tested (Table II).

Hemodynamic observations (Table III, Figure 1)

The cardiac output was measured 30 times in the resting state (Figure 2) by at least two different methods, one of which was always the Fick method (Table III). Output determinations were repeated on subsequent days. Regardless of the technique used, whether measured before or after exercise, with or without sedation, cardiac outputs averaged twice as high as our laboratory normal value estimated under similar conditions. The values ranged from 4.7 to 8.7 L. per minute per M.^{2} with an average of 6.0 (S.D. ± 1.1 L.) (Figure 2), while our normals ranged from 2.5 to 4.7 L. per minute per M.^{2} with an average of 3.5 L. per minute per M.^{2} (S.D. ± 0.6 L.).[5] In T. M. the first elevated cardiac output was measured in 1946 and was still increased when measured again 13 years later. In A. N. and R. R. elevated outputs were recorded in 1957 and in 1959. Observations were made during attempted sleep in five patients. Cardiac output remained unchanged in three but fell from 6.7 to 4.3 L. per minute per M.^{2} and from 5.3 to 2.6 L. per minute per M.^{2} in the other two patients (Figure 3). Normally, sleep results in a 40 per cent reduction in cardiac output (13). The arteriovenous difference was narrowed at rest to 60 per cent of normal. The oxygen consumption was 25 cc. (or 15 per cent) higher than our average normal

[5] The standard error of the difference between the means is 0.27 indicating that the difference is significant and unlikely to have arisen by chance.

value. The heart rate at rest (79 per minute) was normal. The resting stroke volume was twice normal. Pulse pressure at rest was somewhat wider than that of the laboratory normal but was associated, nevertheless, with a normal systolic ejection period for the cardiac rate. Systolic hypertension was noted clinically in four patients and directly recorded in five of the eight patients. Systemic vascular resistance was reduced to approximately 70 per cent of normal, reflecting the peripheral vasodilatation.

Exercise observations (Figure 4)

Six patients were exercised for a four minute period. Oxygen consumption rose an amount equal to or exceeding that of our normal patients, but was met by *increased* oxygen extraction and continued deliverance of the already elevated (but unchanged) cardiac output. The cardiac rate rose normally on effort with a concomitant *fall* in stroke volume. Blood pressure response on effort was similar to the normal, and systemic vascular resistance, already reduced at rest, changed little. This latter pattern simulated the normal dynamics during effort (Figure 1 and 4) and the overall change from rest dynamics to exercise dynamics simulated that seen in patients with acute transient anxiety subjected to exercise (1).

Special studies—Cardiac catheterization

By means of multiple diagnostic techniques, a left to right shunt at all levels within the heart and great vessels was ruled out in each patient. A high venous oxygen content was observed in both the superior and inferior venae cavae indicating that the vasodilatation was diffuse. Their similarity was additional evidence against a large vessel arteriovenous shunt.

Mild mitral stenosis—calculated mitral valve area = 2.5 cm.^{2} (14)—was detected in one pa-

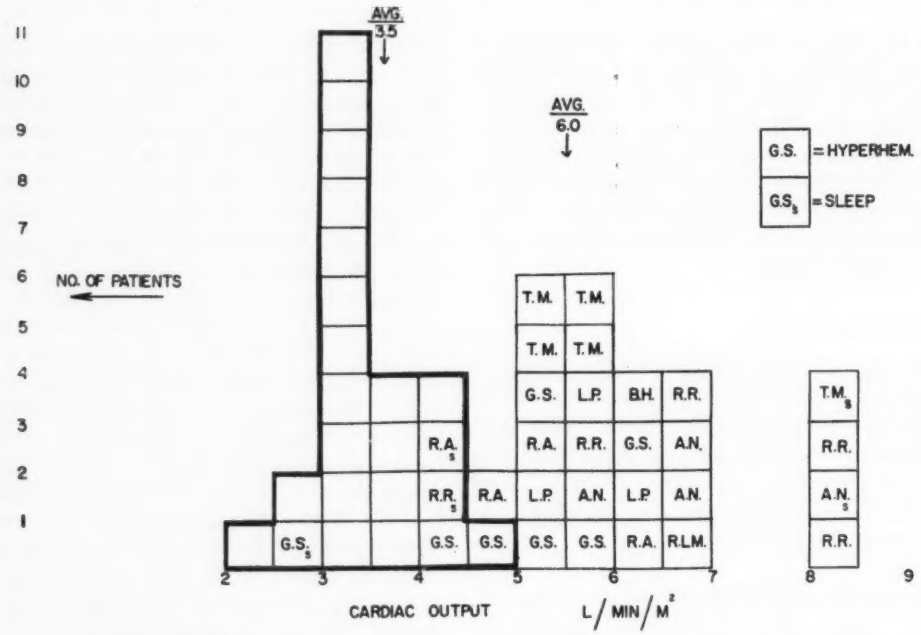

FIG. 2. HEAVILY OUTLINED AREA ENCOMPASSES CARDIAC OUTPUTS DETERMINED AT REST IN 23 NORMAL INDIVIDUALS

The average value was 3.5 L. per minute per M.^{2}. Each box containing initials represents a single determination of cardiac output in a patient with high cardiac output. The small "s" is an output estimated during sleep. The average for the group was 6.0 L. per minute per M.^{2}.

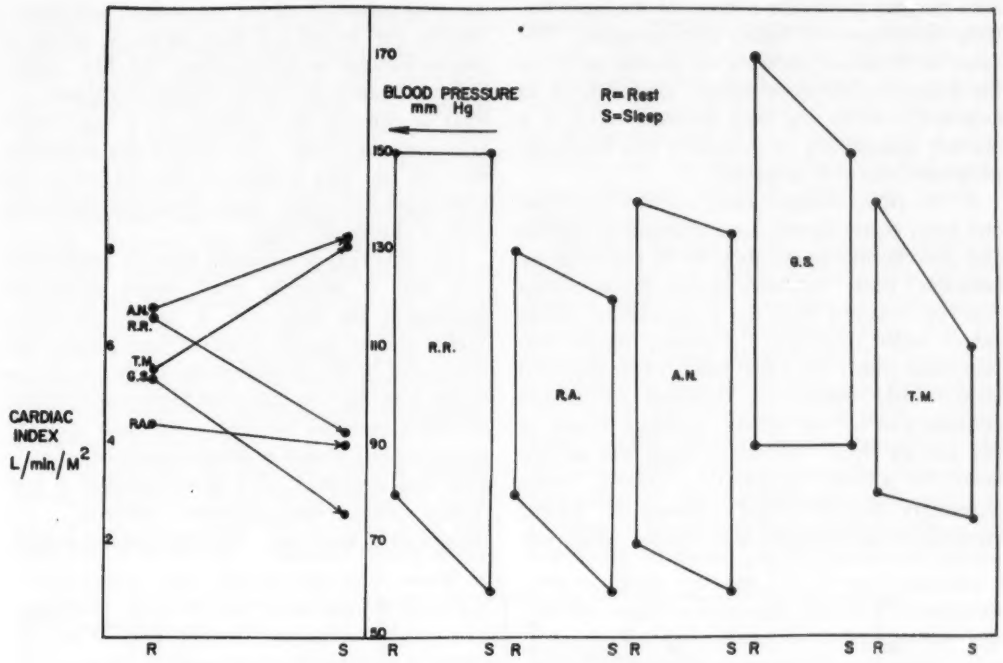

FIG. 3. EFFECT OF SLEEP OR PROLONGED REST IN QUIET DARKENED ROOM ON CARDIAC OUTPUT AND BLOOD PRESSURE

While the blood pressure tended to decrease in all, the cardiac output showed a variable response. In only one patient, however, did output fall to a normal value during sleep. Changes in pulse rate are not shown, as they were normal at rest.

tient (B. H.) and was markedly aggravated by the extremely high rate of blood flow. As a result, pulmonary capillary pressure was elevated to 25 mm. Hg. In Patient A. N. there was evidence for early left ventricular failure as indicated by an increased exercise pulmonary capillary pressure in the absence of mitral stenosis. Pulmonary hemodynamics were normal in the other six patients.

Blood chemical studies (Table II)

Arterial oxygen saturation, pCO_2 and pH were within normal limits; blood lactate and pyruvate and catechol amine levels were normal at rest and on effort. Thyroid studies were normal in all eight patients. Circulating blood volume was within normal limits in six of the seven patients studied. The elevated volume in A. N. may be partly a consequence of associated heart failure.

DISCUSSION

Decreased peripheral resistance is apparently of major etiological significance in most hyperkinetic states as is demonstrated by the opening and closing of an arteriovenous fistula (15–17). On the other hand, to maintain perfusion pressure, the primary resistance fall must be accompanied either by an increased venous return or neurohumoral cardiac stimulation in order for the secondary rise in output to occur (18).

When sympathetic neurohumoral stimulation is the mechanism of the hyperkinetic state, similar coordinated adjustments occur between heart and periphery. As the contractility increases with increased systolic emptying per beat, there is a peripheral vasodilatation particularly in the muscle and splanchnic vascular beds. This could be primary to the sympathetic discharge or secondary to

baro-receptor moderator reflexes as the heart initially discharges at a higher pulse pressure. Because we do not as yet know the precise nature of the syndrome herein presented, it is difficult to ascertain whether the high cardiac output is a primary abnormality or secondary to a decreased peripheral vascular resistance.

In this group of eight young males, intracardiac and great vessel shunts, hyperventilation, acidosis and pheochromocytoma seem to be ruled out by laboratory tests. Evidence against thyrotoxicosis was best obtained from the protein-bound iodine values, rather than from the resting oxygen consumptions which were not measured in the basal state. Mild beriberi is excluded on clinical grounds and by the normal pyruvate values at rest and on effort. Similarly, there was no evidence for arterial unsaturation, anemia, severe pulmonary disease, Paget's disease or hepatic cirrhosis in any of these individuals. Blood volume was normal in all but one patient, and hypervolemia *per se* has not been shown to induce a *chronic* increase in cardiac output (19, 20). Physical examination and X-ray served to exclude, insofar as one can, the possibility of a large vessel arteriovenous fistula. Also against this possibility was the fact that venous oxygen saturation for both upper and lower venae cavae was similar as well as abnormally high.

The following possibilities must be considered. The first is multiple small vessel aneurysms throughout the body, *i.e.*, a variant of Osler-Weber-Rendu disease. None of these patients had cherry-red spots, telangiectasia or hemangiomas of any sort, nor was there any history of epistaxes or gastrointestinal bleeding. Although unlikely, this possibility cannot be totally disproved. Holmgren and associates (21) have described a syndrome called "vaso-regulatory asthenia." Although all of their cases had high cardiac outputs

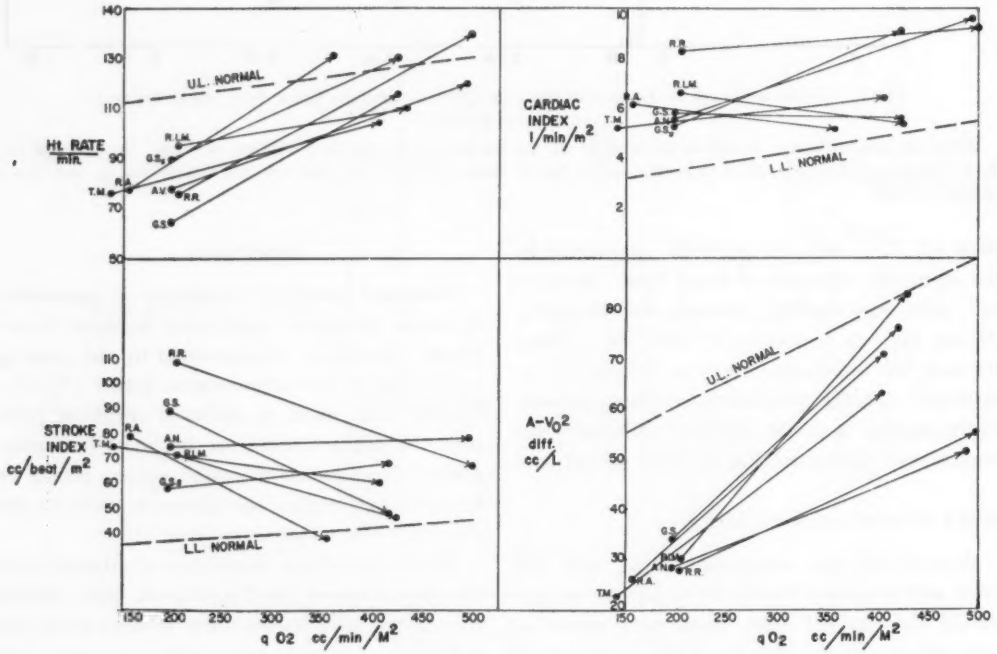

FIG. 4. EFFECT OF EXERCISE ON HEMODYNAMICS

UL = upper limit; LL = lower limit. Note the abnormally high stroke volume at rest which *decreased* to a normal figure for exercise. Cardiac rate was normal at rest and increased in normal fashion on effort. The elevated resting cardiac output generally remained unchanged on effort due to reciprocal changes in rate and stroke output. Oxygen extraction by the tissues, narrow at rest, widened normally on effort.

and narrow arteriovenous oxygen differences, the patients also had marked subjective symptoms of severe fatigue, precordial pain, heart consciousness, breathlessness and dizziness. In comparison, none of our patients had similar symptoms. On the other hand, none of their patients had cardiac murmurs or ventricular hypertrophy, and lungs and heart size were normal on X-ray examination. Whereas the abnormalities reported by Holmgren and co-workers reverted to normal after a period of vigorous physical training, four of our patients were in good physical training at the time of the study. Neurocirculatory asthenia likewise was excluded by lack of symptoms of fatigue, and normal exercise oxygen consumption and lactate levels (2, 22).

Acute *transient* anxiety can produce hemodynamic alterations identical with those seen in our patients (1, 23). Even the fact that heart rates were almost invariably normal and resting oxygen consumptions only slightly elevated would not necessarily rule out the acute anxiety state (1). On the other hand, sedation had no effect on our patients, and sleep caused a diminution in cardiac dynamics in only two cases and relatively minor changes in blood pressure. No conclusions can be drawn from the normal catechol amine levels because the relationship between acute and chronic anxiety states and amine values has not as yet been clearly defined (24, 25). Finally, the multiplicity of studies by simple technics, the persistence of abnormalities over one to 13 year periods, and *other* evidence of *cardiac involvement* militate against these observations reflecting merely an acute *transient* state. On the other hand, a chronic, persistent or frequently recurrent, easily provoked anxiety state could lead to just this situation. It is noteworthy that each of these individuals showed an effect generally characterized as "anxious." They were invariably hard-working and tense, both during laboratory tests and in the performance of their normal daily routines, and had both labile blood pressure and positive cold pressor tests.

It seems probable that a new "set" has been applied to circulatory regulation such that either "resting" output is chronically elevated or the system over-reacts to ordinarily insignificant stimuli. This may be related to an unusual autonomic response to anxiety or to unexplained central and autonomic nervous dysfunction. Rushmer and Smith (26) have shown that stimulation of a specific site in the dog's midbrain can evoke the complete "exercise response" with elevation of cardiac output and rate. Whether such a center exists in man, and therefore results in high cardiac output through improper regulation, cannot be stated at present.

Whether the persistently elevated output noted during all of our studies remains so at all times outside the laboratory situation, or is intermittent, is unknown. The striking fact remains that, contrary to the usual anxiety state, the hyperactive circulation in these patients occurred in association with other objective evidence of cardiac involvement: cardiac murmurs, cardiac overactivity, electrocardiographic evidence of cardiac hypertrophy and occasional gross enlargement and pulmonary plethora. Other high cardiac output states, patent ductus arteriosus, thyrotoxicosus and arteriovenous fistula (presumably through elevation of cardiac output), eventually lead to left ventricular hypertrophy and ultimately to congestive heart failure. Whether the same is true here is not known. This group may constitute another disease entity characterized by a cardiac output chronically elevated above normal, and this elevation and associated systolic hypertension may lead to or be associated with signs of cardiac damage. Certainly, long duration of the high blood flow state is indicated by T. M. and evidence of ultimate functional impairment is shown in Patient A. N.

Hyman (27), in a report on asymptomatic heart disease in 350 young men, includes 26 patients, all of whom had a point of maximal impulse outside the midclavicular line or below the fifth intercostal space or both. Seven of 11 with electrocardiographic abnormalities had left axis deviation in the limb leads. At the other end of the spectrum, anatomically proved cardiac hypertrophy of unknown etiology in adults has been reported by Levy and Rousselot (28) and others (29–34). Some of these patients at least, particularly from Hyman's series of young men, may represent stages in the natural history of a disease process in which the anatomic abnormality noted is an expression of the same hyperkinetic state found in our patients.

SUMMARY

Eight patients have been studied who had in common the following clinical features: a precordial systolic murmur; hyperkinetic heart and arteries; ventricular hypertrophy by electrocardiogram; and, frequently, pulmonary plethora by X-ray.

Hemodynamic observations revealed peripheral vasodilatation and a persistently elevated cardiac output. This latter finding was confirmed on 30 separate determinations including rest, sedation and sleep in selected patients. Cardiac catheterization and other pertinent studies failed to reveal evidence for the usual causes of the high output state.

The association of a high output state with cardiac murmur, cardiac hyperactivity and hypertrophy suggests the possibility of a distinct clinicophysiologic syndrome.

ACKNOWLEDGMENTS

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A STUDY IN MAN OF CEREBRAL BLOOD FLOW AND CEREBRAL GLUCOSE, LACTATE AND PYRUVATE METABOLISM BEFORE AND AFTER EATING*

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It has been shown that subjective feelings of hunger (1) and even objective phenomena such as gastric hunger contractions (2) can be correlated with a decreased arteriovenous glucose difference across the forearm. This decrease in arteriovenous difference of glucose was thought to indicate a decreased rate of glucose utilization by peripheral tissues and its role in hunger regulation has recently been reviewed by Mayer (3, 4). Since the brain is particularly dependent on glucose for metabolism it would be of interest to determine its response to the fasting state and the ingestion of a test meal. If, in the fasting state, such decreased utilization of glucose occurs in the brain as does peripherally, it would be of interest to know what other carbohydrate substances are utilized and particularly whether or not variation in the amount of glucose taken up by the cerebral tissue is related to the sensation of hunger.

MATERIAL AND METHODS

Eight subjects were studied after a 14 hour period of fasting. The patients were selected from the general hospital wards and although they presented various forms of disease (rheumatic heart disease, hypertensive heart disease and anxiety tension states) they had no known intracranial disease and were of normal body weight. In some subjects a cardiac catheter was advanced through the left antecubital vein, the left internal jugular vein and into the jugular bulb. In others the jugular bulb was punctured percutaneously. The catheter or needle was then attached to a manifold to permit rapid sampling. A Cournand needle was placed in a peripheral artery and attached by plastic tubing to a heparin filled manifold for rapid sampling and to a strain gage for pressure recording by way of a Sanborn Poly-Viso. Mean pressures were determined by electrical integration of the pressure curve. Arterial and internal jugular venous blood content of glucose, lactate and pyruvate were determined, respectively, by the methods of Folin and Malmros (5), Friedemann and Haugen (6), and Barker and Summerson (7). Arterial and cerebral venous blood specimens were analyzed for oxygen and carbon dioxide content by the Van Slyke-Neill manometric technique. Cerebral blood flow was determined by the nitrous oxide method of Kety and Schmidt (8).

In each subject cerebral blood flow was determined in the fasting state as were the glucose, lactate and pyruvate in the arterial and internal jugular venous blood. The subject was then fed a standard breakfast consisting of two slices of toast (each covered by one pat of butter), a glass of milk, and a small glass of orange juice containing one heaping teaspoonful of sugar. This meal was estimated to contain approximately 60 Gm. carbohydrate, 15 Gm. protein, and 20 Gm. fat. Determinations of glucose, lactate and pyruvate were made in the arterial and internal jugular venous blood at intervals of 15, 30, 45 and 60 minutes after completion of this meal. Cerebral blood flow was determined again between 30 and 45 minutes after the test meal.

Calculation of cerebral consumption of glucose, lactate and pyruvate in the control period was done by multiplying the arteriovenous differences by the control cerebral flow. Since the 15 minute determination of these substances was drawn halfway between the two determinations of cerebral blood flow, the average of these two observations was used for calculating glucose consumption at this time. The calculation of carbohydrate consumption at 30, 45 and 60 minutes was done assuming the cerebral blood flow had remained the same at each of these periods, and that the flow at each interval was that which was measured between 30 and 45 minutes after the consumption of food. Errors introduced by these assumptions and averages should be small, since there was so little change in the blood flow between the first and second determinations.

RESULTS

Results of this study are summarized in Tables I and II. It will be noted that there were no significant changes in cerebral blood flow, vascular

* This research was supported in part by grants from the Wisconsin Heart Association, the National Heart Institute of the United States Public Health Service, and the Wisconsin Alumni Research Foundation.

resistance, oxygen consumption or respiratory quotient. Peripheral arterial blood pressure, pulse rate, hemoglobin, hematocrit and arteriovenous difference for oxygen and carbon dioxide were also remarkably stable.

Although there was a considerable increase in the arterial level of glucose, the internal jugular glucose content increased also, so that the change in the arteriovenous difference remained small. During the time when the arterial glucose level was rising, the arteriovenous difference increased transiently. As the peak of the curve was reached the arteriovenous difference returned to normal and then as the arterial level began to fall the arteriovenous difference narrowed transiently. Finally, at the end of the study, it again returned to the control state. (See Figure 1.) The calculated glucose consumption increased somewhat but not significantly at 15 minutes, and at 45 minutes was less than during the control observations ($p < 0.05$), even though the blood sugar content remained high at this time compared to the control observations. The arteriovenous difference for lactate and pyruvate indicated that in the fasting state the brain gave up both of these substances to the blood stream. After feeding, the blood level of both of these materials increased and they were taken up by the brain. The increased lactate uptake proved transitory (see Table II) with neither extraction ($r = 0.20$) nor calculated consumption ($r = 0.21$) significantly related to the arterial lactate level. The pyruvate extraction and calculated consumption remained increased throughout the period of study and were correlated positively with the arterial pyruvate level ($r = 0.70$, $p < 0.001$ and $r = 0.68$, $p < 0.001$, respectively). No difference was apparent between those who stated that they were hungry and those who were not.

TABLE I

Cerebral hemodynamic observations before and after eating

	Before	After
Mean arterial blood pressure (mm. Hg)	92 ± 16*	90 ± 17
Arterial oxygen content (ml./100 ml. of blood)	16.9 ± 1.5	17.0 ± 1.5
Arterial-internal jugular O_2 difference (volumes %)	6.5 ± 1.8	6.5 ± 1.5
Cerebral blood flow (ml./100 Gm. brain/min.)	52 ± 12	55 ± 14
Cerebral oxygen consumption (ml./100 Gm./min.)	3.3 ± 0.6	3.5 ± 0.7
Cerebral respiratory quotient	0.98 ± 0.06	0.96 ± 0.07

* Standard deviation (in each case).

TABLE II

Blood carbohydrate changes across the brain before and after eating

	Control	15 min.	30 min.	45 min.	60 min.
Arterial glucose content (mg./100 ml.)	106 ± 7.1*	144 ± 19.8	150 ± 18.8	143 ± 23.3	137 ± 18.0
Cerebral A-V† glucose difference (mg./100 ml. blood)	11 ± 2.3	13 ± 2.5	10 ± 2.7	8 ± 4.6	10 ± 2.7
Cerebral glucose extraction (mg./100 Gm. brain/min.)	5.5 ± 1.1	7.1 ± 2.2	5.5 ± 1.2	4.0 ± 2.1	5.6 ± 1.8
Per cent arterial glucose extracted	10.4	9.0	6.7	5.6	7.3
Arterial lactate content (mg./100 ml.)	5.9 ± 2.0	9.6 ± 4.2	11.6 ± 4.8	13.3 ± 4.5	12.2 ± 3.1
Cerebral A-V lactate difference (mg./100 ml. blood)	−0.5 ± 0.9	0.6 ± 1.3	0.5 ± 0.3	−0.2 ± 0.8	0.4 ± 1.0
Cerebral lactate extracted (mg./100 Gm. brain/min.)	−0.23 ± 0.37	0.36 ± 0.19	0.29 ± 0.16	−0.08 ± 0.43	0.15 ± 0.37
Arterial pyruvate content (mg./100 ml. blood/min.)	1.06 ± 0.32	1.74 ± 0.82	2.11 ± 0.63	2.25 ± 0.52	2.22 ± 0.47
Cerebral A-V pyruvate difference (mg./100 ml. blood)	−0.06 ± 0.11	0.14 ± 0.21	0.16 ± 0.08	0.15 ± 0.23	0.24 ± 0.08
Cerebral pyruvate extraction	−0.31 ± 0.05	0.09 ± 0.13	0.09 ± 0.07	0.09 ± 0.12	0.13 ± 0.06

* Standard deviation (in each case).
† Arteriovenous.

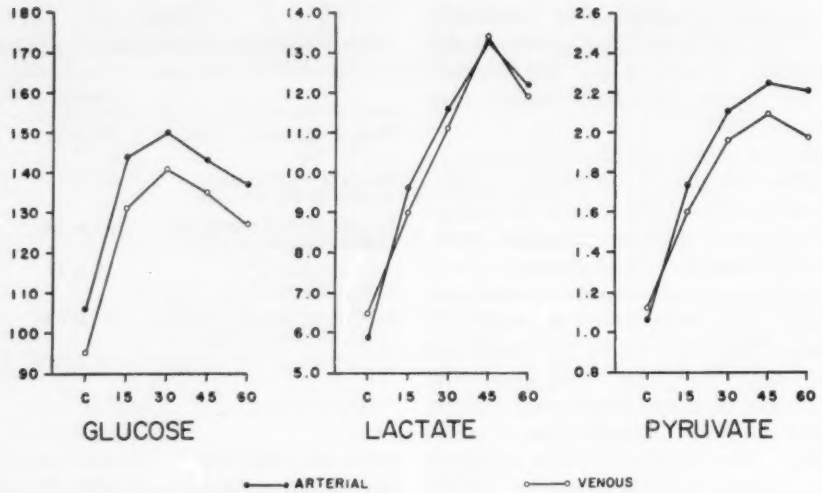

FIG. 1. MEAN ARTERIAL AND INTERNAL JUGULAR BLOOD CONTENT OF GLUCOSE, LACTATE AND PYRUVATE FOR EIGHT SUBJECTS

Values are plotted at the control determination (C) and at each 15 minute interval throughout the ensuing hour. The vertical scale is expressed in milligrams per 100 milliliters of blood.

DISCUSSION

Previous reports of brain glucose, lactate and pyruvate extraction in the fasting state agree reasonably well with those reported here and indicate that, whereas glucose is taken up by the brain in the fasting state, both lactate and pyruvate are given up to the perfusing blood (9, 10). Postprandially, however, the state changes, with glucose consumption fluctuating above and below the control values, and lactate uptake fluctuating above the fasting level, while pyruvate was taken up consistently by the brain and in amounts which correlated directly with the arterial pyruvate level ($r = 0.70$, $p < 0.001$) (see Table II).

Contrary to the behavior of peripheral tissues (1–4) the brain extracts roughly the same quantity of glucose in the fasting state as it does during the peak of the arterial concentration of glucose after a test meal. The percentage of glucose extracted decreases as the arterial blood glucose content rises and the calculated amount of glucose taken up by the brain varies, indicating a transient increase as the level rises and a compensatory decrease as the arterial level falls. Overall, there was no correlation between the arterial glucose level and either the arteriovenous difference across the brain ($r = 0.03$) or the glucose consumption of the brain ($r = 0.08$). These observations are consistent with Gey's report of a very low concentration of glucose in the brain (11) and the hypothesis that the blood to brain gradient of glucose is great enough at all times during physiological conditions for the brain cells to extract what glucose they need. The sustained glucose uptake by the brain and the relative independence of this uptake from the arterial level are also in line with studies of Morgan and Park on the glucose transport into the heart muscle (12). These workers concluded that a transport mechanism rather than simple diffusion explained glucose entrance into the heart muscle cell, since the rate of uptake was not related linearly to the gradient of glucose across the membrane and since there was competitive inhibition of glucose transport by high concentrations of arabinose. If a transport mechanism accepted the major portion of glucose extracted by the brain, but there was transient passive diffusion of glucose into the brain or its interstices during the time that blood sugar rises rapidly, the excess of glucose taken up by the brain during the increase in arterial blood sugar might be expected to be utilized dur-

ing the time when blood sugar fell again to normal. From data concerning glycogen content of the dog brain it has been extrapolated that the human brain contains 1.5 Gm. glycogen (13), hence some storage of glucose seems reasonably certain.

Comparison of the calculated cerebral oxygen consumption and the quantity of oxygen required for complete oxidation of the glucose taken up by the brain (corrected by subtracting the glucose equivalent of the lactate and pyruvate given up to the blood stream by the brain) indicates that in the fasting state the measured oxygen consumption constitutes 88 per cent of that which would theoretically be required. This differs by 12 per cent from the theoretical complete stoichiometric relations found through similar calculations by Himwich and Himwich (10) and may well be due to experimental error.

The theoretical oxygen consumption, as calculated considering carbohydrate taken up and released at each 15 minute interval, averaged 80 per cent of the oxygen consumption calculated through multiplying the arteriovenous oxygen difference across the brain by cerebral blood flow, although fluctuations from 60 to 117 per cent occurred at the lowest and highest points. The data would fit with the hypothesis that the cerebral metabolism of carbohydrate remains essentially unchanged throughout, but that there is transitory carbohydrate accumulation during a time of rising blood level and that this tends to be utilized during the time when the blood carbohydrate level is falling sharply. The fact that these adjustments fluctuated above and below but returned to their control state at the end of one hour seems to deny their relation to hunger and is compatible with Stunkard's report of cessation of hunger contractions in a decerebrate man after glucose administration (14). Furthermore, it seems probable from brain-destructive lesions in experimental animals, produced by gold thioglucose, that the "hunger center" itself may actually be a very small portion of the ventromedial hypothalamus (15, 16). A considerable variation in the quantity of glucose consumed by such a small center might be physiologically important, yet not demonstrable in such gross metabolic measurements as the carbohydrate consumption of the brain as a whole.

CONCLUSIONS

1. A group of eight human subjects has been studied by determination of cerebral blood flow and brain carbohydrate uptake before and after a test meal.

2. During the time when the arterial level of glucose, lactate and pyruvate was rising, the brain took up larger quantities of these carbohydrates. As the peak blood concentration of glucose was reached the uptake returned to the control values, and during the time when the level of the substances was falling rapidly, uptake of glucose by the brain was reduced to below the control value.

3. The fact that glucose and lactate uptake are not related to their absolute arterial level is compatible with the thesis that the arterial level of these substances does not control their rate of utilization by the brain.

4. Brain pyruvate extraction and calculated consumption were correlated directly with the arterial level of pyruvate.

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PHYSICOCHEMICAL AND IMMUNOLOGIC STUDIES OF GAMMA GLOBULINS OF NORMAL HUMAN URINE *

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Normal human urine has been shown to contain up to 150 mg. of protein in a 24 hour aliquot. Electrophoretic analyses of these proteins by a number of observers (1–3) have demonstrated the presence of components corresponding to each of the five major fractions present in serum. In general, however, the resolution of these peaks has been poor, and significant amounts of protein migrating more rapidly than albumin have been demonstrated. Immunoelectrophoretic studies of normal human urine by Grant (4) and Patte, Baldassaire and Loret (5) have revealed the presence of at least eight components which reacted with an antiserum to normal human serum and frequently gave reactions of identity with the corresponding proteins present in serum. Among the proteins identified by these observers were albumin, four α-globulins, two β-globulins and γ-globulin (4).

In spite of the qualitative similarities of the serum and urine proteins, quantitative differences in the relative amounts of the major fractions have been demonstrated. Thus, whereas albumin is the major protein component of serum, the globulin fractions predominate in urine (1, 3). The presence in urine of γ-globulin at a relative concentration equal to and frequently greater than that in serum cannot be explained by current concepts concerning the origin of urinary proteins by a process of glomerular filtration of serum proteins (6, 7) followed by a nonselective tubular reabsorption (8). Such a mechanism would lead to a preferential excretion in the urine of proteins of low molecular weight and would result in an increase in their concentration relative to that of the larger proteins. Since the molecular weight of the major fraction of γ-globulin in serum is approximately two and one-half times that of albumin, the presence in urine of relatively greater amounts of protein with the mobility of γ-globulin suggested the existence of additional protein species which have not been detected in serum. The present study presents evidence for the existence in normal urine of proteins with a mobility similar to γ-globulin and an approximate molecular weight of 35,000, and which are antigenically closely related to the γ-globulin fraction of serum. While this work was in progress, low molecular weight γ-globulin fractions have also been demonstrated in normal human urine by Webb, Rose and Sehon (9).

MATERIAL AND METHODS

Twenty-four hour urines were collected from 12 normal males and stored in the cold at 4° C. Following filtration to remove formed elements and insoluble precipitates, the urines were dialyzed against running tap water for 24 hours in the cold and concentrated to a volume of 10 to 15 ml. by pervaporation at 0 to 4° C. In a number of instances protein was precipitated by the addition of 650 Gm. ammonium sulfate to each liter of urine. Following centrifugation at 3,000 rpm for one hour, the precipitate was dialyzed against running tap water to remove the salt, and the protein solution was concentrated as above. Both methods yielded similar results. For large scale physicochemical studies, 5 to 10 L. urine from a single subject were processed.

Electrophoresis. This was performed using starch or polyvinyl chloride resin 427 (Goodrich Chemical Company) as the supporting medium and barbital buffer, pH 8.6, $\mu = 0.05$ or 0.10 as described by Kunkel (10). Protein was eluted from one-half inch segments by displacement filtration and its concentration estimated by the modified Folin tyrosine method (11). Fractions corresponding in mobility to the γ-globulin peak of a control serum separated on the same block were pooled and concentrated by ultrafiltration through collodion membranes [1] with a vacuum of approximately 260 mm. mercury.

Ultracentrifugation. This was done in a Spinco Model E ultracentrifuge at a temperature of 20° C. us-

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[1] Schleicher and Schuell Company, Keene, N. H.

ing cells with double sector centerpieces (12). Quantitative measurements were carried out with a comparator as described by Trautman (12). Molecular weights were determined by the technique of Archibald (13) at speeds of 20,410 to 24,630 rpm. The partial specific volume ($\bar{V}$) for the urinary γ-globulin was assumed to be similar to that of serum γ-globulin and equal to 0.74.

Immunochemical studies. Antisera to Fraction II γ-globulin (Lederle C-380) and to the urinary γ-globulin fraction from two normal subjects were prepared in rabbits by the subcutaneous route with Freund adjuvant. The antiserum against Fraction II had previously been shown to be specific for the fraction of γ-globulin with a sedimentation coefficient of 7S (7S γ-globulin) (14). Antigens were examined by double diffusion in 0.5 to 1.0 per cent agar according to the method of Ouchterlony (15), by immunoelectrophoresis by the method of Williams and Grabar (16) and by quantitative precipitin analysis as previously described (14). The amount of protein precipitated was determined by the modified Folin technique (11) and converted to milligrams of nitrogen according to a standard curve prepared with human γ-globulin. Supernate analyses with 7S γ-globulin were carried out in capillary tubes and by double diffusion in agar.

Radioactive studies. In order to determine the source of the γ-globulin present in urine, studies were performed with I^{131}-labeled human γ-globulin. Fraction II γ-globulin (Lederle C-543) was iodinated by standard methods (17).[2] The iodinated material was passed through a Dowex-1 ion-exchange column to remove free iodide and through a Seitz filter and cultured for sterility before use. The resultant material contained less than 2 per cent nontrichloracetic acid (TCA)-precipitable radioactivity; 50 to 100 μc. of this I^{131}-labeled γ-globulin with a specific activity (SA) of 10 μc. per mg. of protein was given by intravenous injection to four normal male subjects. Urines were collected for the first eight and following 16 hours, and serum was drawn eight and 24 hours following injection. The urines were dialyzed for 48 hours against running tap water to remove free iodide, concentrated by pervaporation and separated with the corresponding sera by starch zone electrophoresis. Radioactivity on the recovered fractions was assayed in a well-type scintillation counter with a sensitivity of 0.9×10^6 counts per minute (cpm) per microcurie of I^{131} above a background of 150 cpm.

[2] We are indebted to Drs. M. Rothchild and M. Oratz of the Veterans Administration Hospital, New York, N. Y., for their assistance in carrying out this phase of the study.

RESULTS

Representative electrophoretic patterns obtained from one normal urine and the corresponding serum are shown in Figure 1. Compared with the distinct resolution of the major protein components obtained in the serum pattern, the peaks in the urine appeared to be broader and more nonhomogeneous, thus making it difficult to define individual components. In the majority of urines examined, the concentration, relative to the other components, of the protein fraction with the mobility of γ-globulin was greater than in the corresponding serum. In general the urines contained significant amounts of protein with a

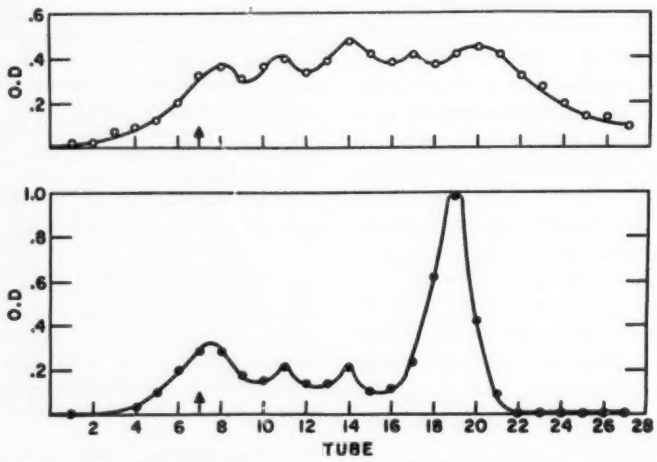

FIG. 1. ELECTROPHORETIC PATTERNS OF ONE NORMAL 24 HOUR URINE SPECIMEN (ABOVE) AND SIMULTANEOUSLY RUN SERUM (BELOW)

Separation carried out in starch, barbital buffer, pH 8.6, $T/2 = 0.05$.

A B C

FIG. 2. ULTRACENTRIFUGAL PATTERNS OF γ-GLOBULINS OBTAINED FROM URINES OF THREE DIFFERENT SUBJECTS

Pictures were taken after 80 minutes at a speed of 52,640 rpm. Sedimentation proceeds from left to right.

mobility greater than albumin. This fraction could not be detected in the sera under the conditions of this study.

Figure 2 shows the ultracentrifugal patterns of the proteins migrating with the mobility of γ-globulin obtained from the urines of three different subjects. Two of these (A and C) were prepared by pervaporation, while the middle one (B) was obtained by ammonium sulfate precipitation. In each instance there was a major broad nonhomogeneous peak with an approximate $s_{20,w}$ of 1.5–1.6S.[3] In a number of instances there were small amounts of a second more rapidly sedimenting peak with an approximate $s_{20,w}$ of 7S. In the γ-globulin fractions prepared from normal urines this additional component, which appears to correspond to the 7S γ-globulin of serum, never exceeded 10 per cent of the total protein present. In the urines of five patients with glomerulonephritis, pyelonephritis and nephrosclerosis whose urinary protein excretion amounted to 5 to 10 Gm. per day, this more rapidly sedimenting component increased in absolute and relative concentration and constituted more than 75 per cent of the total γ-globulin fraction.

Table I lists the $s_{20,w}$ at a protein concentration of 0.1 to 0.3 per cent of the urine γ-globulins of eight subjects. Observed sedimentation coefficients were corrected to water as a solvent and expressed in Svedberg units. Because no appreciable effect of concentration on the $s_{20,w}$ was found, the values given are uncorrected for protein concentration. The observed $s_{20,w}$ in seven of the subjects ranged from 1.1 to 1.9S, with a mean of 1.6S. One subject (A.L.) had an observed $s_{20,w}$ of 2.5 and 2.6 on two occasions. Nine different samples were examined from one subject (E.F.) and the sedimentation coefficient varied from 1.2 to 1.9S, with a mean of 1.6S.

In order to rule out the possibility that this slowly sedimenting peak was a result of protein breakdown during the prolonged preparative procedures, control experiments were performed: Cohn Fraction II γ-globulin which did not contain detectable amounts of protein sedimenting more slowly than the 7S peak, or 1 to 2 ml. normal serum, was diluted in 2,000 ml. normal saline or normal urine and handled in a manner identical to a simultaneously prepared urine. There was no evidence of selective loss of the 7S peak or breakdown of this peak to the low molecular weight component during handling. Similarly, addition of pepsin, plasmin and streptokinase to sera or γ-globulins under these conditions did not result in breakdown of 7S γ-globulin to smaller fractions.

Molecular weights were calculated during the "approach to sedimentation equilibrium" by the method of Archibald (13). The values of four different samples from one subject ranged from 23,800 to 38,000 and are listed in Table I. Because most of the preparations examined were not completely homogeneous, the significance of the one low value cannot be fully evaluated. Since this technique initially emphasizes the heavy components of a polydisperse solution, it appears possible that the lower value was due to the greater purity of this particular preparation. However, the possibility also exists that this may represent an even smaller fraction of γ-globulin than the

TABLE I

Sedimentation coefficients and molecular weight determinations of urine γ-globulins

Subject	No. of examinations	$s_{20,w}$*	Molecular weight
E. F.	9	1.6 (1.2–1.9)†	
O. S.	1	1.1	
M. F.	1	1.1	
S. I.	1	1.7	
B. A.	1	1.7	
A. L.	2	2.6 (2.5–2.7)†	
S. N.	1	1.8	
A. N.	1	1.8	
E. F.	1	1.6	30,000
E. F.	1	1.7	38,000
E. F.	1		35,600
E. F.	1	1.6	23,800

[3] Sedimentation coefficient in water at 20° C., expressed in Svedberg units (S).

* Sedimentation coefficient in water at 20°C. expressed in Svedberg units (S).

† Mean and (range).

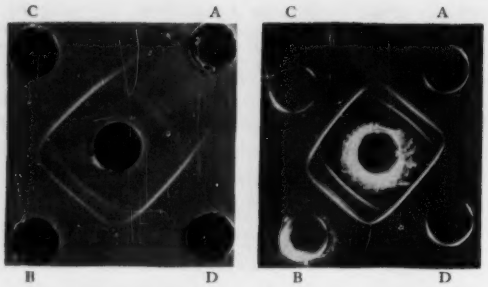

FIG. 3. DOUBLE DIFFUSION IN AGAR OF URINE γ-GLOBULINS (A AND B) AND SERUM 7S γ-GLOBULIN (C AND D) AGAINST AN ANTISERUM TO SERUM 7S γ-GLOBULINS

three samples whose molecular weights are distributed around a mean of approximately 35,000.

Immunological studies

When tested with an antiserum to normal human γ-globulin by the capillary precipitin technique, the urine component with the mobility of γ-globulin invariably gave a precipitate at the interphase. However, in general, the precipitate was slower in appearance and finer in consistency than that obtained with the homologous γ-globulin from serum.

Figure 3 illustrates the reactions obtained when urine γ-globulins and serum 7S γ-globulins react in agar with an antiserum prepared against Cohn Fraction II γ-globulin. In all instances only a single sharp line appeared between the antiserum and the serum γ-globulin component. In the case of the γ-globulins prepared from urine, two or three lines were usually present. The line closest to the antigen well showed a reaction of complete identity with a line formed by the 7S γ-globulin and probably represented small amounts of this protein frequently detected also by ultracentrifugation. In only one specimen (Figure 3A), which was ultracentrifugally free of 7S material, was this line absent. All of the urine specimens showed the presence of one or two additional lines which had diffused further from the source of antigen. These lines never crossed the 7S line and appeared to show a reaction of partial identity with it. In several preparations of urine γ-globulin (wells A and B) there was no change in the curvature or position of these lines following a 10-fold increase in the 7S γ-globulin fraction, thus suggesting that they were indeed due to smaller, more rapidly diffusing substances which cross-reacted with the 7S component.

In order to exclude the possible presence in Fraction II of small amounts of low molecular weight components similar to those isolated from urine, and to obtain preparations of "pure" 7S

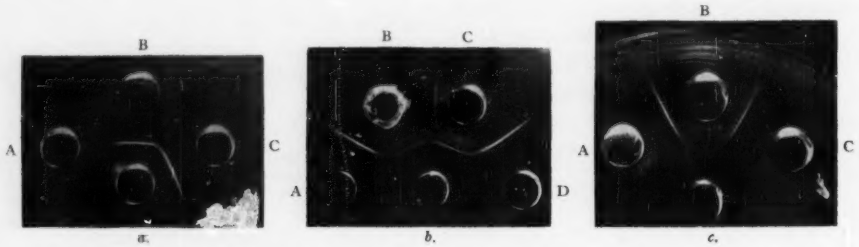

FIG. 4. *a.* DOUBLE DIFFUSION IN AGAR COMPARING REACTION OF AN ANTISERUM TO URINE γ-GLOBULIN (CENTER WELL) WITH URINE γ-GLOBULIN (A), 7S SERUM γ-GLOBULIN (B), AND WHOLE SERUM (C)

b. DOUBLE DIFFUSION IN AGAR COMPARING REACTION OF URINE γ-GLOBULIN (CENTER WELL) AND 7S SERUM γ-GLOBULIN (A AND D) WITH ANTISERA TO SERUM 7S γ-GLOBULIN (B) AND TO URINE γ-GLOBULIN (C)

There is fusion of all of the 7S γ-globulin lines and one of the more rapidly diffusing lines present in the urine.

c. DOUBLE DIFFUSION IN AGAR COMPARING REACTION OF URINE γ-GLOBULIN (CENTER WELL) WITH AN ANTISERUM TO URINE γ-GLOBULIN ABSORBED WITH 7S γ-GLOBULIN (A); SAME ANTISERUM UNABSORBED (B); SAME ANTISERUM ABSORBED WITH WHOLE SERUM (C)

The lines between the antisera represent a reaction of the antisera with the excess γ-globulin used in absorption.

FIG. 5. IMMUNOELECTROPHORETIC ANALYSIS OF γ-GLOBULIN FROM URINE (CENTER) AND COHN FRACTION II (TOP AND BOTTOM)

Two additional lines with a γ mobility are present in the urine. The anode is to the right.

γ-globulins free of any possible lighter contaminants, several preparations of Fraction II were separated into eight to 10 fractions by centrifugation in a saline density gradient. The details of this procedure have been published previously (18). The time of centrifugation was such that the bottom 0.5 ml. was free of albumin ($s_{20,w}$ = 4S) which had been added to a simultaneously separated control preparation, and therefore presumably also free of lighter molecules. The top two fractions were rich in albumin and should also have contained any possible lighter contaminants. When tested with an antiserum to 7S γ-globulin, both the top and bottom fractions gave only a single line which fused to give a reaction of identity. Absorption of this antiserum with equivalence amounts of the more rapidly sedimenting fraction ("pure" 7S γ-globulin), removed all antibody activity to the urinary proteins.

Confirmatory evidence was obtained with antisera to urinary γ-globulin fractions. In double diffusion studies in agar only a single line appeared with whole serum or 7S γ-globulin while two or three additional lines were present when urinary γ-globulins were tested (Figure 4*a*). The position and curvature of these additional lines suggested that they were due to low molecular weight substances. When the reaction of the urine γ-globulins was compared with antisera to urine and serum γ-globulins, a reaction of identity was observed between the outer 7S lines and one of the more rapidly diffusing lines (Figure 4*b*). The outer line also fused with a line formed by 7S serum γ-globulin and each of the antisera. This would suggest that some of the antibodies in both antisera reacted with at least two common antigens in the urine. Only one line, the most rapidly migrating one in Figures 4*a* and 4*c*, appeared to be unique to the urine since it could not be removed by repeated absorptions with 7S γ-globulins or whole serum (Figure 4*c*). This may represent a constituent specific to urine, possibly a mucoprotein. One line did not resolve sufficiently to permit an adequate comparison.

A group of representative patterns obtained by immunoelectrophoresis using the same antiserum

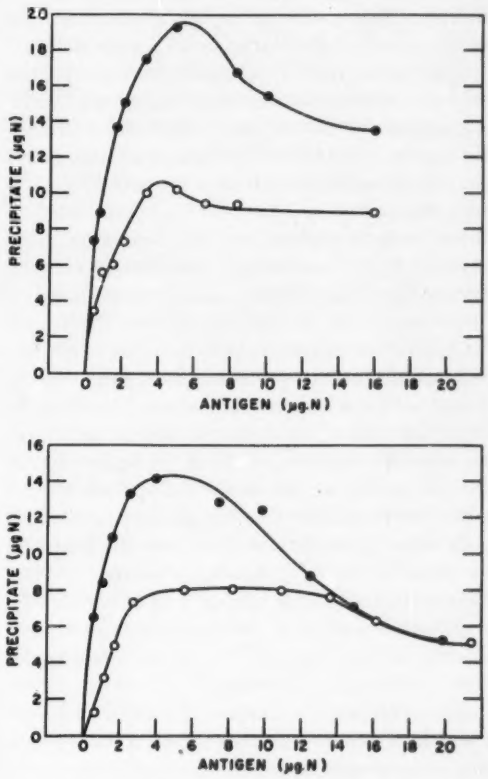

FIG. 6. QUANTITATIVE PRECIPITIN CURVES OBTAINED WITH AN ANTISERUM TO HUMAN γ-GLOBULIN COMPARING REACTION OF TWO URINE γ-GLOBULIN FRACTIONS WITH 7S γ-GLOBULIN FROM SERUM

●——● serum γ-globulin. ○——○ urine γ-globulin.

TABLE II

Total radioactivity and specific activities of protein fractions of urine and serum from one subject after the administration of I^{131}-labeled gamma globulin

Material	Time	γ-Globulin			β-Globulin			α_2-Globulin			Albumin		
		Protein	Total activity	Specific activity	Protein	Total activity	Specific activity	Protein	Total activity	Specific activity	Protein	Total activity	Specific activity
	hrs.	*mg.*	*cpm*	*cpm/mg. protein*	*mg.*	*cpm*	*cpm/mg. protein*	*mg.*	*cpm*	*cpm/mg. protein*	*mg.*	*cpm*	*cpm/mg. protein*
Serum	8	26.2	13,620	520	8.3	266	32	8.6	56	6.5	34.9	89	2.5
Serum	24	27.0	8,675	320	5.5	159	29	7.8	89	11.5	22.8	89	3.9
Urine	0–8	16.2	2,980	184	19.9	745	23.5	28.0	425	15.2	34.5	1,475	42.5
Urine	8–24	21.5	6,450	299	27.0	1,420	52.5	20.0	466	23.3	34.0	1,480	43.5

to human 7S γ-globulin is shown in Figure 5. Cohn Fraction II γ-globulin gave only a single line of precipitation with the broad distribution characteristic of γ-globulin. The urinary fraction again demonstrated two additional lines in the γ-globulin region with a mobility slightly greater than that of the simultaneously separated 7S γ-globulin.

Quantitative precipitin studies were carried out with an antiserum to normal human γ-globulin. Figure 6 shows two representative sets of precipitin curves, each of which compares the reaction of the γ-globulin prepared from urine with 7S γ-globulin from serum. The urine γ-globulin used in these studies contained less than 5 per cent of 7S material by ultracentrifugal analysis. In each instance the homologous antigen precipitated a larger amount of protein than the urine γ-globulin. In general the curve obtained with the urine fraction had a broader equivalence zone than was obtained with the serum γ-globulin. The decrease in the amount of precipitate, observed with similar amounts of either protein in the region of antigen excess in each set of curves, appears to preclude the possibility that the precipitate obtained with urine γ-globulin was due to the small amount of contaminating 7S γ-globulin present. When the supernates of these curves were tested with 7S γ-globulin in a capillary precipitin tube and by agar double diffusion studies, no antibody could be detected beyond the equivalence point in any of the curves. These observations would indicate a very great degree of cross-reaction between the γ-globulins of serum and urine.

I^{131}-labeled γ-globulin

Fifty to 100 μc. of I^{131}-labeled γ-globulin was given intravenously to four normal male subjects. Prior to and following iodination this material did not contain detectable amounts of material sedimenting more slowly than 7S γ-globulin on ultracentrifugal examination, and gave only a single line with an antiserum to 7S γ-globulin. Urines were collected for the first eight hours and from the eighth to the twenty-fourth hour, dialyzed against running tap water for 48 hours to remove all traces of free iodide, concentrated by pervaporation and fractionated by starch zone electrophoresis. The results in each of the four subjects were similar in that approximately two-thirds of the radioactivity were associated with the urinary γ-globulin fraction, while only approximately 10 per cent migrated with the albumin fraction.

The data from one representative subject are listed in Table II. Eight and 24 hours following the intravenous administration of 75 μc. of I^{131}-labeled γ-globulin, the bulk of the radioactivity in serum was associated with the γ-globulin fractions with a mean specific activity of 420 cpm per milligram protein. The γ-globulins prepared from the urine collected during the same period did not demonstrate a 7S peak on ultracentrifugal examination. Sixty-five per cent of the total nondialyzable protein-bound radioactivity during the second collection period was associated with the fraction of γ mobility, while only 15 per cent migrated with the albumin. The specific activity of the γ fraction was approximately 299 cpm per milligram protein, which is somewhat lower than that of the serum γ-globulin at 24 hours.

In contrast to these observations are the results of control studies with nonprotein-bound labeled iodide. I^{131} was added *in vitro* to normal serum. The mixture was then dialyzed for 48 hours and subjected to starch zone electrophoresis

in a manner similar to the urines. In three experiments the major fraction of the radioactivity was dialyzable, and only 0.4 to 2.5 per cent of the radioactivity was recovered with the starch block eluates. Of this fraction only 0.02 to 0.9 per cent was associated with proteins having a mobility of γ-globulin, while 66 to 75 per cent migrated with the albumin fraction and 18.5 to 22.0 per cent was located ahead of the albumin peak. More than two-thirds of the radioactivity associated with the albumin in these experiments appeared to be firmly bound, since it was precipitated by trichloroacetic acid and could not be removed by further dialysis. The specific activity of the albumin in these experiments was significantly greater than that in the albumin recovered from the urine. Because of these differences in the distribution of the radioactivity noted after the *in vivo* administration of I^{131}-labeled γ-globulin and after the *in vitro* addition of iodide to serum, it seems likely that in the *in vivo* studies virtually all of the radioactivity with the mobility of γ-globulin was protein-bound. On the other hand, some of the radioactivity migrating with the albumin may have represented free iodide. Since the latter cannot be accurately quantified by the techniques employed, the fraction of the total protein-bound radioactivity associated with the γ-globulin fraction was probably significantly greater than the figure of 65 per cent calculated above.

These observations favor the view that under the conditions of this study, the bulk of the protein-bound radioactivity in urine and serum was associated with the fraction of γ mobility, and that the 7S γ-globulin in serum gave rise to the corresponding fractions in urine. While these experiments do not completely eliminate the presence in the iodinated globulins of low molecular weight material, in amounts too small to be detected by the techniques employed, which could then be selectively excreted by the kidney, this possibility does not appear probable. It seems likely that such proteins would be handled by the kidneys in a manner similar to the Bence-Jones proteins which are of approximately the same size. These molecules are rapidly excreted during the first 12 hours after their injection (19). Under these circumstances, the specific activity of the urine γ-globulins would be expected to be higher than that of the corresponding serum fractions, and not lower as found in these studies.

DISCUSSION

The present study demonstrates the presence in normal urine of proteins with the mobility of γ-globulin which are antigenically closely related to the serum γ-globulins, but differ from them in being only one-fifth to one-sixth their size. Qualitatively, similar findings have recently been reported by Webb, Rose and Sehon (9). However, the molecular weight reported by these observers is of the order of 10,600. These discrepancies and the wide range of sedimentation rates and molecular weights noted in the present study, would suggest the presence of a broad spectrum of breakdown products of different sizes derived from γ-globulin, rather than the existence of a single protein. Evidence for the presence of a number of components is further suggested by the broadness of the ultracentrifugal peak, and the presence of at least four precipitin lines in an antiserum to urine γ-globulin. Similarly, carbohydrate analyses on a number of fractions proved to be extremely high and variable, and would suggest the presence of some carbohydrate-rich contaminants, possibly similar in nature to the carbohydrate-rich material remaining at the origin reported by Rigas and Heller (1).

The results of isotopic studies reported here as well as those by Webb, Rose and Sehon (9) offer strong evidence that the urine γ-globulins are derived from the corresponding serum fractions. These studies would further suggest that in man there exists a mechanism of glomerular filtration of serum proteins similar to that demonstrated in a number of animal species by Sellers, Smith, Marmorsten and Goodman (20), Oliver, MacDowell and Lee (21) and Spector (22). Since the concentration of the γ-globulin fraction in urine relative to the other components is frequently greater than in serum, it seems probable that these fragments are formed prior to glomerular filtration and that, by virtue of their small size, they pass through the glomeruli more readily than albumin. A similar conclusion is also suggested by the appearance of larger amounts of 7S γ-globulin, without a concomitant rise in the output of the smaller fragments, in pathologic states associated with an in-

creased glomerular permeability. However, it has not been possible to date to demonstrate the presence of these components by ultracentrifugal and immunologic means in the sera of seven patients wtih acute renal shutdown of four to 10 days' duration, in whom an accumulation of these proteins would appear likely. The possibility that some of these proteins are added to the urine after completion of the process of glomerular filtration, or that they may originate in tissues of the urinary tract cannot be excluded with absolute certainty from these studies.

The exact nature and significance of these proteins is not fully understood at present. Although they resemble the pathologic Bence-Jones proteins somewhat in size, they have none of the other specific physicochemical properties characteristic of these proteins. Similarly, the isotopic studies have demonstrated that these normal urine γ-globulins appear to be derived from serum γ-globulin, whereas the Bence-Jones proteins appear to be synthesized independently (19). Recent work by Porter (23) has demonstrated that papain can break down normal rabbit γ-globulin into three types of smaller fragments. One of these retains the antigenic constituents characteristic of γ-globulin. Breakdown of human γ-globulin by similar procedures has also been observed to yield two antigenically distinct fragments (24). It seems possible that the urine γ-globulins described here represent some of the natural end products of γ-globulin metabolism *in vivo* corresponding to these fractions produced *in vitro*. The relationship of these two types of γ-globulin breakdown products is currently under study.

SUMMARY

1. The major constituent of the γ-globulin fraction of normal human urine appears to be one-fifth to one-sixth the size of the serum γ-globulin, and has a sedimentation coefficient of approximately 1.6S.

2. This low molecular weight fragment of γ-globulin is antigenically closely related to serum 7S γ-globulin.

3. Isotopic studies have demonstrated that it is primarily derived from the serum γ-globulin fraction.

4. The possibility that the fraction represents a natural breakdown product of γ-globulin and its bearing on current theories of glomerular filtration is discussed.

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STATIC VOLUME-PRESSURE RELATIONS OF EXCISED LUNGS OF INFANTS WITH HYALINE MEMBRANE DISEASE, NEWBORN AND STILLBORN INFANTS

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The clinical features of hyaline membrane disease have been reviewed recently (1, 2). When the disease is fatal the lungs at autopsy are usually grossly atelectatic, suggesting the appearance of liver. Microscopically, this atelectasis is seen to be patchy and widespread; scattered through the lungs are dilated terminal bronchioles and alveolar ducts that are lined with an eosinophilic membrane.

The significance of the membrane during life is uncertain. Behrle, Gibson and Miller (3) demonstrated at autopsy that the expansion of lungs of infants was impeded by the membrane. More recently, Craig, Fenton and Gitlin (4) showed from latex casts of the airways that the membrane may cause obstruction. But Gruenwald (5) has taken the view that the membrane may be only "a secondary phenomenon that appears in a previously diseased lung." He has stressed instead the greater importance of the atelectasis to which these lungs appear vulnerable.

Our object was to study the static elastic behavior of lungs containing hyaline membranes. For this purpose static volume-pressure relations of lungs from three sources were compared: newborn infants with a histologic diagnosis of hyaline membrane disease, newborn infants dying of other causes, and stillborn infants. On the basis of these measurements, estimates were made of the differences in functional residual capacity (FRC) and pulmonary compliance that might have prevailed during life among the first two groups; these estimates were compared with the few data available on living infants (6, 7). The factors that may be responsible for changes in elastic behavior of lungs with hyaline membrane disease will be discussed.

METHODS

The material included 10 lungs from newborn infants with hyaline membrane disease, five lungs from stillborn infants and five lungs from infants who died within the first four days of life. The right or left lung alone was used in each experiment, the other side being reserved for examination by the pathologist. All measurements were made within 48 hours of the time of death. Before study the tissue was kept at temperatures of about 5 to 10° C. to prevent autolysis.

The preparation is shown schematically in Figure 1. The lung was suspended from a cannula tied into the main bronchus. As the burette was elevated fluid flowed into the intermediate bottle displacing gas into the lung. The difference between levels of fluid in the burette and the bottle represented the distending pressure applied to the lung. This pressure was read from a water-filled manometer connected to the bronchial cannula. The

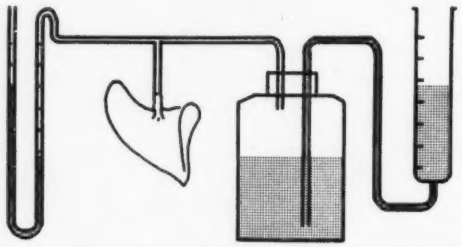

FIG. 1. APPARATUS FOR INFLATION AND DEFLATION

Inflation and deflation were carried out in fixed steps of pressure. Airway pressure (the difference in level between the burette and the intermediate bottle) was measured by means of the fluid-filled manometer on the left. Correction was made for compression of the gas in the intermediate bottle and the tubing leading to the lung.

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volume of air entering the lung was read directly from the burette; correction was made for compression of gas in the system by the distending pressure. The lung was inflated in fixed steps of pressure until a peak level of 35 cm. H_2O was reached. The lung was then deflated, usually in the same steps, until zero airway pressure was reached. During inflation and deflation two minutes were allowed for changes of volume at each pressure; at the end of this time equilibrium was virtually established.

The lung was weighed at the outset; its volume was then measured by displacement of fluid before and after each run. Some of the lungs from newborn infants contained a small amount of gas at the outset. This volume of gas was estimated, assuming that the specific gravity of the tissue was 1.065 (8). To correct for leaks that may have occurred during the procedure, the change in volume of the lung following each run was compared with the change in level of fluid in the burette; when the loss of fluid from the burette was greater than the increase in volume of the lung, the discrepancy was ascribed to a leak. A correction was made assuming that the rate of leakage was directly proportional to airway pressure. With one exception, the leaks represented less than 12 per cent of the peak volume in the lung; for the one exception, the leak equaled 28 per cent.

RESULTS

The weight of the infants and of the lungs, the duration of extrauterine life, and the diagnoses are listed in Tables I through III. The three groups showed no significant differences in body weight or lung weight. To calculate the ratio of lung-to-body weight, the assumption was made that the right and left lungs, only one of which was available for study, were equal in weight. Had the right lung represented as much as 60 per cent of the total weight of the lungs, the change in values for this ratio would have been negligible.

TABLE I

Physical characteristics of five stillborn infants having diagnosis of intrauterine asphyxia

No.	Sex	Body weight*	Lung studied	Lung weight†	$\frac{\text{Lung weight‡}}{\text{Body weight}} \times 100$
		Kg.		*Gm.*	%
1	M	0.67	R	6	1.8
2	M	2.20	L	22	2.0
3	M	2.25	L	25	2.2
4	F	2.62	L	18	1.4
5	M	3.07	L	28	1.8
Mean		2.16		20	1.8

* At autopsy.
† Weight of single lung which was studied.
‡ Based on estimated weight of entire lung (see text).

At a maximal distending pressure of 35 cm. H_2O the lungs of newborn and stillborn infants appeared fully inflated, whereas inflation of the lungs with hyaline membranes was incomplete. The surface of the latter lungs showed multiple lobulations surrounded by depressed, unexpanded segments of tissue. Photographs of sections from a lung with hyaline membranes are shown in Figure 2A through D). Figure 3 and Table IV compare the average static volume-pressure relations of the lungs of the three groups of infants. To permit comparison of lungs of different size, volume was expressed as milliliters of gas per gram of tissue. At a pressure of 35 cm. H_2O, the lungs of newborn infants contained about four times as much gas per unit of weight as the lungs of infants with hyaline membrane disease. The value for lungs of stillborn infants was intermediate. These differences were roughly similar over most of the volume-pressure curve.

TABLE II

Extrauterine age, physical characteristics and diagnosis of five newborn infants

No.	Sex	Age	Body weight*	Lung studied	Lung weight†	$\frac{\text{Lung weight‡}}{\text{Body weight}} \times 100$	Diagnosis
		hrs.	*Kg.*		*Gm.*	%	
1	M	57	0.88	R	13	3.0	Prematurity
2	F	70	1.60	R	18	2.2	Pneumonia
3	F	72	1.65	R	22	2.7	Pneumonia
4	M	24	2.44	L	23	1.9	Intraventricular hemorrhage
5	M	72	2.56	L	18	1.4	Hydronephrosis
Mean			1.82		19	2.0	

* At autopsy.
† Weight of single lung which was studied.
‡ Based on estimated weight of entire lung (see text).

TABLE III

Extrauterine age and physical characteristics of 10 infants having hyaline membrane disease

No.	Sex	Age	Body weight*	Lung studied	Lung weight†	Lung weight‡ / Body weight × 100
		hrs.	*Kg.*		*Gm.*	*%*
1	M	10	0.85	R	10	2.4
2	M	24	1.85	R	19	2.0
3	M	22	1.95	L	17	1.7
4	M	48	2.10	L	25	2.4
5	M	7	2.35	R	26	2.2
6	M	29	2.38	L	24	2.0
7	M	20	2.40	L	21	1.8
8	F	36	2.60	L	29	2.2
9	M	70	2.80	R	30	2.1
10	M	43	3.00	R	26	1.7
Mean			2.29		23	2.0

* At autopsy.
† Weight of single lung which was studied.
‡ Based on estimated weight of entire lung (see text).

DISCUSSION

The study has shown that excised lungs containing hyaline membranes contain less gas per unit of weight than do the lungs of newborn or stillborn infants of similar size. This is true for all inflating pressures between 0 and 35 cm. H_2O. As a corollary, the slope of the volume-pressure curve of the lungs with hyaline membranes is reduced over virtually the entire range of volume. That similar conclusions may be inferred about these lungs during life is suggested by the work of McIlroy and Christie (9) who found that the distensibility of human lungs was of the "same order as that found during life" in specimens studied up to 72 hours after death. In our study the upper limit of time following death was 48 hours.

There is evidence suggesting that the lungs of infants and children may become more distensible (contain more gas per unit of lung weight for a given distending pressure) with advancing age (10, 11). It is therefore important to stress that no significant difference in gestational age was present among the three groups, using body weight as an index of age.

The lungs of newborn infants, in spite of the presence of pneumonia in two specimens, were generally about twice as distensible as those of stillborn infants (Figure 1, Table IV). The reason for this difference is not clear; it was not revealed by the pathologist's examination of the tissue.

We have given emphasis to volume-pressure relations during deflation following application of a fixed peak distending pressure. Such measurements are less variable than measurements made during inflation, especially if inflation proceeds from the passively collapsed state. In the latter circumstance the presence of fluid in the airways (12), the configuration of the airways (13), and to some extent the interval of time following death (11) may affect the volume-pressure relations.

FRC and associated transpulmonary pressure

Estimates were made of the average differences in FRC and compliance that might have been present during life between the infants with hyaline membrane disease and the newborn infants. To do this, the data in Figure 3 were replotted in Figure 4 with the vertical axis expressing absolute volume without relation to the weight of the lungs and the horizontal axis expressing transpulmonary pressure. Such estimates have no application to stillborn infants and were not made.

Berglund and Karlberg (6) reported measurements of FRC in healthy newborn infants. Their subjects with one exception were heavier than the newborn infants in our study. Based on the correlation they found between FRC and birth weight, the average FRC of our newborn infants without hyaline membrane disease would be expected to fall between 60 and 70 ml. For this range of

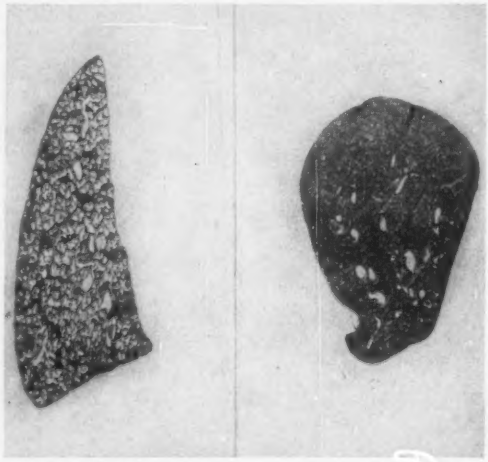

FIG. 2A FIG. 2B

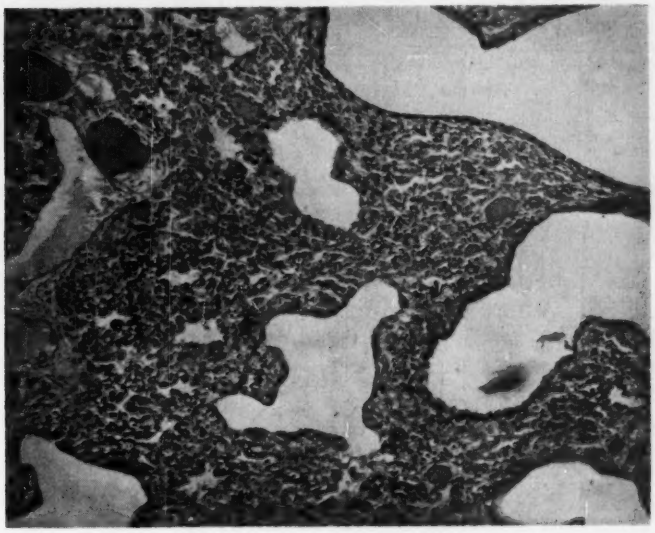

FIG. 2C

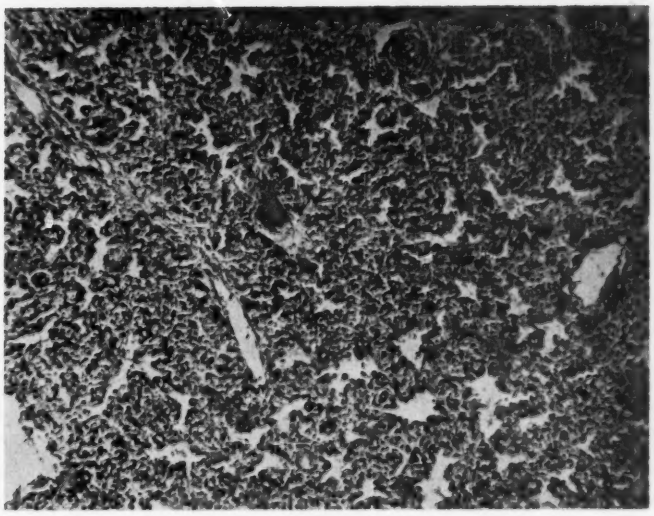

FIG. 2D

FIG. 2. SECTION OF LUNG CONTAINING HYALINE MEMBRANES FROM AN INFANT WEIGHING 1.67 KG.

A shows the specimen inflated with air to a pressure of about 35 cm. H_2O and fixed with Bouin's solution; B shows a section from the same lung fixed at atmospheric pressure; C is a photomicrograph of the lung in A (140 × magnification). Note the irregular filling: some areas are overdistended and others are unopened. D is a photomicrograph of the lung in B (140 × magnification). Note the widespread atelectasis.

TABLE IV

Static volume-pressure characteristics during deflation of excised lungs of stillborn infants, newborn infants, and infants dying of hyaline membrane disease

	No.	Body weight*	Volume Pressure (cm. H_2O) 35	25	15	10	5	2.5	0
		Kg.	*ml./Gm.*						
Stillborn	1	0.67	1.3	1.2	1.1	1.1	1.0	0.9	0.1
	2	2.20	1.0	1.0	0.9	0.8	0.7	0.6	0.1
	3	2.25	1.8	1.7	1.7	1.6	1.3	1.1	0.2
	4	2.62	1.6	1.5	1.4	1.3	1.2	0.9	0.3
	5	3.07	1.2	1.2	1.1	0.9	0.8	0.8	0.2
Mean		2.16	1.4	1.3	1.2	1.1	1.0	0.9	0.2
Newborn	1	0.88	1.1	1.0	1.0	0.9	0.8	0.7	0.2
	2	1.60	2.7	2.6	2.5	2.3	1.9	1.3	0.3
	3	1.65	1.7	1.7	1.6	1.4	1.2	1.0	0.2
	4	2.44	2.9	2.8	2.6	2.5	2.3	1.9	0.4
	5	2.56	4.4	4.4	4.1	3.9	3.5	2.6	1.2
Mean		1.82	2.6	2.5	2.4	2.2	1.9	1.5	0.5
Hyaline-membrane	1	0.85	0.4	0.4	0.4	0.3	0.2	0.1	0.1
	2	1.85	0.8	0.7	0.5	0.3	0.1	0.0	0.0
	3	1.95	0.4	0.4	0.3	0.2	0.1	0.1	0.0
	4	2.10	0.6	0.6	0.5	0.4	0.2	0.2	0.0
	5	2.35	0.9	0.9	0.8	0.7	0.6	0.3	0.0
	6	2.38	0.2	0.2	0.2	0.2	0.2	0.1	0.0
	7	2.40	1.3	1.2	1.2	1.1	0.9	0.7	0.0
	8	2.60	1.2	1.1	1.1	1.0	0.8	0.7	0.0
	9	2.80	0.3	0.3	0.2	0.2	0.1	0.0	0.0
	10	3.00	1.2	1.1	1.0	0.8	0.7	0.5	0.3
Mean		2.29	0.7	0.7	0.6	0.5	0.4	0.3	0.1

* At autopsy.

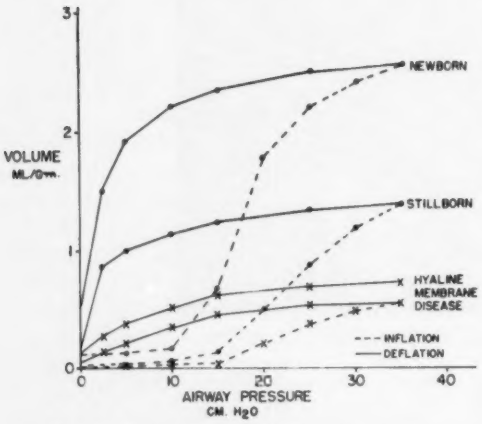

FIG. 3. VOLUME-PRESSURE CHARACTERISTICS

The ordinate refers to gas volume in milliliters per gram of tissue; airway pressure on the abscissa is equivalent to transpulmonary pressure. Four of the lungs with hyaline membrane disease were studied only during deflation and are represented by a separate curve.

volume, referring to Figure 4, the transpulmonary pressure would have been about 4 cm. H_2O, a value not significantly different from the average static end-expiratory esophageal pressure found in healthy, seated young adults (14). Berglund and Karlberg (6) reported that the FRC ranged from 25 to 45 ml. in three newborn infants with clinical and X-ray evidence of extensive atelectasis. If the lungs in our study may be accepted as typical of hyaline membrane disease, at least in its final stage, then a significant reduction of FRC is to be expected. Figure 4 shows that the average volume of gas in these lungs, exclusive of the tra-

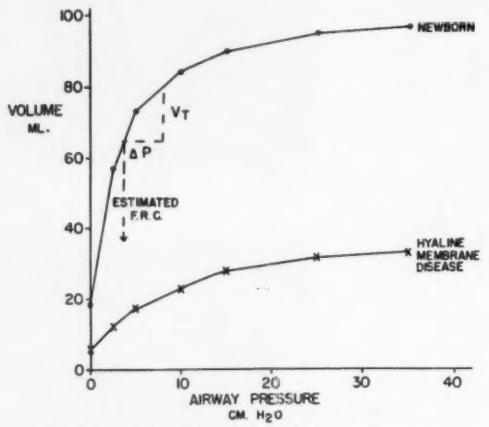

FIG. 4. VOLUME-PRESSURE CHARACTERISTICS

The ordinate refers to total gas volume in milliliters. The estimate of functional residual capacity (FRC) is based on data from Berglund and Karlberg (6). The tidal volume (V_T) is an average value (15 ml.) from data of Karlberg and associates (15). The change in transpulmonary pressure necessary to produce this tidal volume is indicated by ΔP.

chea and proximal airways, was about 35 ml.[1] at a peak pressure of 35 cm. H_2O. For the same pressure, the volume of gas in the newborn lungs was almost 100 ml. Over most of the range of pressure studied, a three- to fourfold difference in volume would have been present, so that the FRC, terminally, in the infants with hyaline membrane

[1] To test whether an appreciable number of uninflated units of the lungs could have been "forced open," pressure was raised to 60 and 80 cm. H_2O in a few preparations. At these levels of pressure there was more extensive inflation of the lungs. The increase in volume, however, was associated with the appearance of leaks through the pleural surface.

disease would be expected to be around 20 ml. (assuming the same transpulmonary pressure for both groups).

Pulmonary compliance

From Figure 4 the difference in compliance during life between the two sets of lungs could be estimated if data were available on tidal volume and either FRC or its associated transpulmonary pressure. For example, if in each group the tidal volume were about 15 ml., a value consistent with the findings of Karlberg and associates (15), and if the end-expiratory pressure were about 4 cm. H_2O, the average values for compliance would have been 3.3 ml. per cm. H_2O for the newborn infants and only 0.8 ml. per cm. H_2O for the infants with hyaline membrane disease.[2] Cook and associates (7) reported a value of 5.2 ± 0.4 ml. per cm. H_2O for a group of healthy newborn infants whose weight at birth averaged 3.1 Kg. These authors were able to measure compliance in two infants considered to have hyaline membrane disease. At the height of the illness, before recovery began, the values were 0.7 and 1.3 ml. per cm. H_2O.

According to our estimates, therefore, the lungs with hyaline membrane disease showed roughly the same magnitude of reduction in FRC and compliance, both values falling to about one-fourth the level of values for the newborn lungs. It is possible, however, that changes in FRC may at times be small relative to the changes in compliance. The reason is that with the appearance of atelectasis, considered an important component of the disease (16, 17), the thoracic cage might be expected to resist reduction of its own volume. The increasing elastic resistance of the thorax would then operate as a force distending the lungs, that is, there would be an increase in end-expiratory transpulmonary pressure (pleural pressure would become more subatmospheric). As a result the volume of units of the lungs remaining open would enlarge in accordance with their individual volume-pressure characteristics. If these units enlarged sufficiently, the slope of their volume-pressure curves, and therefore the slope of the curve for the entire lung, would become depressed.

The means by which the disease affects the elastic behavior of the lungs is not clear. There is no histological evidence that elastic tissue is damaged structurally, or that fibrosis occurs. A change in the surface tension of fluid lining the airways and alveoli has recently been described in this laboratory (18), which offers a basis for the atelectasis that is commonly observed. Whether the hyaline membrane itself acts as a plug completely shutting off sections of the lungs, as suggested by the histologic study of Craig, Fenton and Gitlin (4), or depends for its effect upon changes in surface forces secondary to partial occlusion of the airways or to a combination of these factors, is uncertain. An effort was made to estimate the relative importance of complete and partial plugging in four experiments in the following way: first, volume-pressure measurements were made inflating the lungs with air. The lungs were then freed of air with a method described by von Neergaard (19) in which the lungs were placed in a vacuum jar and the pressure in the jar was lowered to about 15 mm. Hg. Measurements were then repeated substituting saline for air.[3] Saline minimizes surface forces by substituting a fluid-to-fluid interface for the gas-fluid interface; the pressure needed to distend the lungs is thereby reduced (20). Saline filling was not entirely satisfactory, for in each instance there were leaks through the pleural surface, too widespread to be corrected, that appeared at pressures of about 8 to 10 cm. H_2O. We have no explanation for this paradox of leaks occurring during saline studies but not during gas studies when higher distending pressures are used. Despite the presence of leaks, however, some of the lungs appeared to fill more completely with fluid than they had with air; areas of the lungs that had not inflated with air at a pressure of 35 cm. H_2O now appeared to fill at a pressure of only 10 cm. H_2O. The evidence suggests that inflation of the lungs may be

[2] It is recognized that the slope of the static elastic curve obtained on deflation from a peak distending pressure of 35 cm. H_2O is not exactly the same for the next inspiration. Instead, the curve of the next inflation has a lower slope (13). For this reason, the estimates of compliance given here are probably higher than would be found during spontaneous breathing.

[3] The lungs were submerged in a saline bath according to a technique described by Mead, Whittenberger and Radford (13).

prevented at least in part by forces operating at the gas-fluid interface.

For the interface to resist an inflationary pressure as high as 35 cm. H_2O, assuming for the moment that no increase in surface tension has occurred, there must be significant narrowing of the airway owing presumably to the presence of hyaline material. An estimate of the degree to which such narrowing may have progressed can be made from the following relationship developed by LaPlace:

$$P = \frac{2T}{r}$$

where P = the difference in pressure across the gas-fluid surface, T = the surface tension, and r = the radius of curvature of the surface. Normally, nearly all alveolar units are opened by a pressure of 20 cm. H_2O. In the diseased lungs containing partially blocked units that were not opened by pressures of 35 to 40 cm. H_2O (some remained closed at pressures up to 80 cm. H_2O), the radius of curvature of the surface and, therefore, the diameter of the lumen itself must have been reduced to at least half its normal size.

The findings indicate that when hyaline membrane disease is advanced, portions of the lungs will not inflate at transpulmonary pressures as high as 35 cm. H_2O. At this level of pressure there is a possibility that units already open may be damaged.

SUMMARY AND CONCLUSION

Comparison was made of virtually static volume-pressure relations of excised lungs obtained from 10 infants with hyaline membrane disease, five newborn infants dying from other causes, and five stillborn infants. To eliminate the influence of size on these measurements, volume was expressed as milliliters of gas per gram of tissue. Special emphasis was given to the volume-pressure relations during deflation from a peak airway pressure of 35 cm. H_2O. At all pressures the lungs with hyaline membranes contained less gas per unit of weight than the control lungs; in addition, the slope of their entire volume-pressure curve was depressed. From these volume-pressure relations estimates were made of the magnitude of differences in FRC and compliance that might have been present during life. The differences compared closely with direct measurements made by other investigators. The low compliance in membrane-containing lungs was thought to be due to a reduction in number of units participating in ventilation. The relative importance of changes in surface forces that might lead either to a collapse of units or to increased resistance to inflationary pressures and of mechanical plugging could not be determined from these studies.

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INTERRELATIONS OF PLASMA POTASSIUM CONCENTRATION, PLASMA SODIUM CONCENTRATION, ARTERIAL pH AND TOTAL EXCHANGEABLE POTASSIUM *

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Accurate and quantitative definition of the factors that determine plasma potassium concentration has not yet been attained. However, important relationships have been noted between plasma potassium concentration and acid-base disturbances (1–11), acute and chronic losses of body potassium (2, 12) and changes in plasma sodium concentration (13, 14). Conversely, frequent exceptions to these individual relationships are seen. The intimate association between plasma sodium concentration and body sodium, potassium, and water suggests that an analogous multifactored control might obtain for plasma potassium concentration (15). When body potassium content is constant, changes in the plasma potassium level may reasonably be attributed to osmotic and electrochemical influences which alter the concentration gradient of potassium across the cell membrane. The status of intracellular metabolism has a major effect on this gradient, since the anoxic, damaged or glucose-depleted cell leaks potassium at an accelerated rate (16–18).

An analysis of the various determinants of plasma potassium concentration and a consideration of their possible interrelationships are of some importance. Clarification of the nature and degree of any such interrelationships may modify or verify concepts of the physiological mechanisms affecting changes in electrolyte distribution and in addition may facilitate more precise clinical interpretations.

Simultaneous measurements of total exchangeable potassium, total body water, arterial and venous blood pH and arterial and venous plasma electrolyte concentrations were made in a heterogeneous group of chronically ill patients who had been in a steady state [1] for at least two or three days before study. The correlations of plasma potassium concentration with plasma sodium concentration, arterial pH, pCO_2, total exchangeable potassium and various combinations of these measurements were analyzed statistically.

METHODS

Definitions. The symbols and abbreviations used in this paper are defined as follows: K_p, potassium concentration in milliequivalents per liter of arterial plasma water; Na_p, sodium concentration in milliequivalents per liter of arterial plasma water; Cl_p, chloride concentration in millequivalents per liter of arterial plasma; $(CO_2)_p$ total CO_2 content in millimoles per liter of arterial plasma; pCO_2, partial pressure of CO_2 in millimeters Hg in arterial plasma; H_a, hydrogen ion concentration in Moles $\times 10^{-8}$ per liter of arterial blood (to simplify calculation, the factor of 10^{-8} is omitted); K_e, total exchangeable potassium in milliequivalents; TBW, total body water in liters; DBW, dry body weight in kilograms calculated as body weight minus total body water; D_2O, deuterium oxide; THO, tritiated water.

Seventy-four patients were studied. The age, sex, state of hydration, and clinical diagnosis are summarized in Table I. The patients were selected to provide a wide range of plasma potassium concentrations and pH values; the group was also limited to lean or malnourished subjects to eliminate as far as possible the distorting effect of body fat in interindividual comparisons of K_e. In 23 subjects who were seriously ill the investigation was confined to measurements of blood pH and electrolyte concentrations. The group as a whole consisted of 7 patients with various types of heart disease, 15 with cirrhosis of the liver, 14 with renal disease, 21 with pulmonary disease, 3 with gastrointestinal disease, 8 with neurological disease, and 6 with other disorders, including carcinomatosis, barbiturate intoxication, and senility. Thirty-eight of these patients were edematous.

The patients were classified as far as possible accord-

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[1] No significant changes in clinical status, renal or respiratory function or plasma electrolyte concentrations.

ing to the four major categories of acid-base disturbance. Normal pH in this laboratory for arterial blood is 7.43 ± 0.02; the normal $(CO_2)_p$ is 25.6 ± 2.4 mMoles per L. Acidotic patients, *i.e.*, those with arterial pH less than 7.41, were designated under respiratory acidosis if $(CO_2)_p$ was greater than 28.0 mMoles per L., and under metabolic acidosis if $(CO_2)_p$ was less than 23 mMoles per L. Alkalotic patients, *i.e.*, those with arterial pH greater than 7.45, were listed under respiratory alkalosis if $(CO_2)_p$ was less than 23 mMoles per L. and under metabolic alkalosis if $(CO_2)_p$ was greater than 28 mMoles per L. In seven cases in which normal arterial pH was associated with abnormal $(CO_2)_p$ the findings were interpreted in the light of the clinical status. Four patients with unidentifiable acid-base disturbance, together with 16 cases in which data were insufficient and five patients in whom both arterial pH and $(CO_2)_p$ were normal were listed in the unclassified category. No attempt has been made to separate compensated from uncompensated cases. None of the experimental subjects was treated with carbonic anhydrase inhibitors during the period of study.

All subjects were given an analyzed diet containing 10 mEq. sodium and 21 mEq. potassium daily during the period of study. Food and fluid were withheld for the period of D_2O or THO equilibration.

TABLE I

Summary of clinical data

Disease		Male	Female	Age range	Weight range	Transudates
	(*No. of cases*)			*yr.*	*Kg.*	
Heart	(*7*)	6	1	40–74	33–80	7
Kidney	(*14*)	11	3	17–75	39–80	7
Gastrointestinal	(*3*)	2	1	35–40	40–56	0
Liver	(*15*)	7	8	35–86	39–72	13
Lung	(*21*)	19	2	25–82	39–68	5
Neurological	(*8*)	6	2	25–80	42–67	4
Miscellaneous	(*6*)	4	2	40–72	46–50	2

TABLE II

Summary of laboratory data: plasma values

Patient	K_p*	Na_p*	Cl_p*	$(CO_2)_p$*	pH		pCO_2*
					Arterial	Venous	
No.	*mEq./L. plasma water*	*mEq./L. plasma water*	*mEq./L. plasma*	*mMoles/L. plasma*			*mm. Hg*
Metabolic acidosis							
14	4.65	172.5	121.3	20.9	7.37		36.0
17	5.72	140.2	115.8	11.6	7.36		21.5
19	5.32	153.1	118.6	9.8	7.32		20.0
20	4.70	150.1	109.9	16.8	7.40		28.0
25	5.84	143.9	116.5	8.1	7.15		25.0
31	5.12	141.0		10.2	7.28	7.24	23.5
35	6.53	114.7		14.4	7.40	7.36	24.0
37	2.98	154.2		6.9	7.29	7.24	15.0
39	4.76	142.9	105.7	17.6	7.34	7.35	35.5
40	6.16	127.1		18.4	7.35	7.26	35.0
42	4.74	157.2	116.8	6.6	7.15	7.11	19.5
55	3.51	131.6	99.2	15.2	7.40		25.0
56	5.34	146.0	109.0	22.3	7.36	7.33	40.0
61	4.08	148.6	115.0	18.9	7.37	7.36	32.5
66	5.60	135.4	88.8	20.4	7.39	7.33	35.2
69	5.15	143.9	116.6	10.3	7.23		24.5
71	5.48	127.5	98.5	19.7	7.35		36.0
74	4.35	132.8		18.3	7.28	7.30	39.5
Metabolic alkalosis							
5	4.58	141.2	94.8	34.4	7.44		51.0
6	3.55	139.8		29.6	7.55		35.5
9	3.74	161.6	96.7	34.5	7.46		49.0
10	3.08	140.8	92.1	35.8	7.50		46.0
13	5.12	143.2	91.4	30.4	7.47		42.2
15	2.89	139.0	95.2	29.3	7.54		35.5
44	3.11	146.6	101.5	28.1	7.52	7.45	36.0
46	2.34	142.8		33.2	7.56	7.51	38.0
47	2.54	144.0	100.0	30.8	7.49	7.49	41.0
49	2.78	150.7		31.7	7.54	7.50	38.0
54	3.46	135.5	89.4	29.4	7.53	7.47	37.5
62	4.26	146.8	103.0	29.8	7.46	7.42	42.5
63	3.57	158.3	100.0	34.9	7.49	7.44	46.5
72	2.56	134.0	91.8	30.5	7.53	7.49	37.5

* K_p = potassium concentration; Na_p = sodium concentration; Cl_p = chloride concentration; $(CO_2)_p$ = total CO_2 content; pCO_2 = partial pressure of CO_2.

TABLE II—*Continued*

Patient	K_p*	Na_p*	Cl_p*	$(CO_2)_p$*	pH		pCO_2*
					Arterial	Venous	
No.	*mEq./L. plasma water*	*mEq./L. plasma water*	*mEq./L. plasma*	*mMoles/L. plasma*			*mm. Hg*
Respiratory alkalosis							
4	3.95	131.0	96.6	22.6	7.48		30.5
11	4.18	139.5		18.8	7.59		20.0
38	6.15	125.7		15.8	7.48	7.45	23.0
51	5.01	148.5	115.8	17.5	7.54	7.51	21.0
53	1.93	143.9	109.3	14.3	7.51	7.42	19.0
57	2.87	145.6	95.7	22.6	7.48	7.44	33.5
59	4.93	142.1	106.1	20.8	7.47	7.41	29.0
Respiratory acidosis							
1	4.57	144.7		26.3	7.25		59.0
2	4.82	141.9	92.3	32.0	7.37		54.5
3	4.25	146.9	98.3	32.0	7.29		66.0
7	4.37	144.3	99.3	28.5	7.41		47.5
16	4.41	146.2	98.2	33.3	7.43		50.0
41	5.42	140.8	85.1	45.2	7.39	7.37	74.5
52	4.85	143.9	100.0	35.8	7.42	7.37	55.0
58	4.28	141.8	100.0	28.8	7.40	7.29	47.0
70	4.31	142.8	103.5	30.9	7.37		53.0
73	5.58	139.2	101.6	28.6	7.42	7.41	44.5
Unclassified							
8	4.97	140.1	104.2	24.5	7.45		35.0
12	3.58	140.3	106.8	24.2	7.45		35.5
18	4.02	142.5		26.6	7.52		33.5
21	4.52	141.1			7.44		
22	4.50	148.5			7.39		
23	3.78	142.5	98.5		7.48		
24	2.75	134.9			7.49		
26	4.43	141.4			7.44		
27	3.38	132.6	91.8		7.56		
28	4.67	146.6	106.2		7.47		
29	4.28	151.9			7.45	7.42	
30	6.68				7.27	7.25	
32	5.11	137.5			7.44	7.35	
33	4.62	142.9			7.46	7.39	
34	4.42	136.0			7.56	7.52	
36	4.80	139.0		26.4	7.44	7.38	41.0
43	5.00	148.8	105.3	25.3	7.44	7.42	42.0
45	4.12	139.5	101.0	22.2	7.44	7.43	33.0
48	5.20	144.2	100.2	29.1	7.45	7.39	41.5
50	4.87	141.9	97.5		7.39	7.33	
60	3.11	142.5	102.7	25.7	7.48	7.44	35.0
64	2.60	138.6	87.5		7.51	7.46	
65	5.17	141.3	101.0	24.3	7.45	7.42	36.0
67	3.81	136.3	97.7		7.50	7.42	
68	4.62	141.0	104.1		7.38		
Mean	4.34	141.8	101.8	23.8	7.43	7.39	37.1
Range	1.93–6.68	114.7–172.5	85.1–121.3	6.9–45.2	7.15–7.59	7.11–7.52	15.0–74.5

The sequence and technique of isotope administration and collection of urine and blood for isotope assay have been described previously (19, 20). Three hundred to 350 μc. K^{42}, and to a few subjects 1 to 2 mc. tritium, were the maximum doses of administered radioactivity. The periods of isotope equilibration were 40 hours for K^{42} and six hours for D_2O or THO. Blood was drawn almost simultaneously from the femoral artery and antecubital vein at the end of the K^{42} equilibration period and immediately before the deuterium or tritium injection. Glycolysis and clotting in these samples were inhibited by icing and heparin.

The analytical methods for isotope assay have been described previously (19, 21, 22). Sodium and potassium were measured in dilutions of urine and plasma with a lithium internal-standard flame photometer. Plasma water was determined gravimetrically by drying 1 ml. aliquots, delivered from calibrated pipettes, at 104° C. for 72 hours. Plasma chloride was estimated by electrometric titration (23), and total CO_2 content of

TABLE III

Summary of laboratory data: body composition and derived values

Patient	K_e*	K_e/Kg. body wt.	TBW*	TBW/Kg. body wt.	K_e/DBW*	$K_e/DBW \times H_a$	$\frac{K_e/DBW \times H_a}{Na_p}$*
No.	*mEq.*	*mEq./Kg.*	*L.*	%	*mEq./Kg.*		
Metabolic acidosis							
17	2,508	36.8	45.9	67.3	112.5	490.5	3.50
19	2,164	38.5	39.9	71.1	133.6	639.9	4.18
20	1,251	34.6	18.7	51.9	71.9	286.1	1.90
25	2,234	34.6	33.2	51.4	71.4	505.5	3.51
31	2,295	48.8	30.7	65.2	140.8	739.2	5.24
35	2,012	32.2	47.6	76.4	136.9	532.5	4.64
39	1,176	23.6	28.2	55.8	53.5	244.4	1.71
40	2,887	35.9	55.5	69.0	115.9	518.1	4.08
42	1,357	29.8	29.0	63.7	82.2	582.0	3.70
56	2,975	43.1	49.9	72.2	154.9	676.9	4.64
61	1,422	24.6	44.3	76.8	106.1	453.0	3.05
66	1,572	34.2	28.1	61.1	88.1	358.6	2.65
69	1,929	36.1	27.8	52.1	75.5	444.7	3.29
71	1,707	31.8	38.0	70.8	109.2	488.1	3.83
74	2,265	32.1	40.9	57.9	76.4	401.1	3.02
Metabolic alkalosis							
5	2,165	35.8	38.6	63.7	98.4	375.2	2.66
6	1,529	30.7	30.9	62.1	80.9	228.1	1.63
13	1,893	35.9	28.6	54.1	78.1	264.8	1.85
15	1,381	24.0	36.0	62.5	64.2	184.9	1.33
44	1,973	39.6	32.5	65.3	114.2	344.9	2.38
47	1,751	29.9	31.6	53.9	64.9	210.3	1.46
49	1,213	23.0	26.7	50.6	46.7	134.5	0.89
54	2,817	39.3	49.4	69.0	126.9	399.7	2.95
62	1,318	30.9	24.7	57.7	73.0	253.3	1.73
63	2,549	45.2	33.9	60.1	113.2	366.8	2.32
72	1,492	28.7	26.5	51.0	58.5	172.6	1.28
Respiratory alkalosis							
4	1,934	41.7	28.9	62.2	110.3	365.1	2.79
51	869	27.7	20.3	64.8	78.3	225.5	1.52
53	1,623	33.7	28.3	58.7	81.6	252.1	1.75
57	1,725	34.5	29.4	58.7	83.6	276.7	1.90
59	2,261	28.5	49.6	62.4	75.7	256.6	1.81
Respiratory acidosis							
1	1,859	34.4	27.7	51.1	70.3	395.1	2.73
2	2,201	42.1	33.7	64.5	118.3	505.1	3.56
16	1,860	38.8	28.7	59.7	96.4	358.6	2.45
58	2,036	32.7	34.9	55.9	74.2	295.3	2.08
70	1,914	49.0	25.3	64.7	138.7	592.2	4.15
73	1,102	33.2	21.2	63.9	91.8	348.8	2.51
Unclassified							
8	1,092	27.9	28.0	71.7	97.5	346.1	2.47
12	1,887	39.9	27.7	58.6	96.3	341.9	2.44
23	1,054	23.2	25.3	55.5	52.2	172.8	1.21
27	1,469	25.7	28.2	49.3	50.7	139.4	1.05
28	2,214	49.6	30.0	67.5	151.6	513.9	3.51
43	1,306	33.2	26.7	68.0	104.0	377.5	2.53
45	1,338	21.2	39.6	62.7	56.7	205.9	1.49
48	1,894	38.7	26.4	54.0	84.2	292.2	2.03
50	1,203	24.4	28.7	58.1	58.3	237.3	1.67
60	1,545	29.9	32.4	62.7	80.3	265.8	1.87
64	1,162	29.7	20.5	52.4	62.4	192.8	1.39
65	1,518	32.4	27.7	59.1	79.3	281.5	1.99
67	1,607	34.0	26.3	55.5	76.5	241.7	1.77
68	2,049	30.9	33.0	49.7	61.3	255.6	1.81
Mean	1,776	33.7	32.3	60.9	89.8	353.6	2.51
Range	869–2,975	21.2–49.6	18.7–55.5	49.3–76.8	46.7–154.9	134.5–739.2	0.89–5.24

* K_e = total exchangeable potassium; TBW = total body water; DBW = dry body weight; H_a = hydrogen ion concentration; Na_p = sodium concentration.

arterial plasma was determined by Van Slyke's manometric method (24). The pCO_2 was estimated from the Singer-Hastings nomogram (25). Arterial and venous blood pH were measured at 37° C. with a Beckman model GS pH meter, calibrated with buffer standards prepared from high purity salts provided by the National Bureau of Standards. The latter measurements were made within 30 minutes of sampling; previous experiments in this laboratory had indicated that iced specimens showed no change in pH for at least 60 minutes after sampling. All chemical determinations were done in duplicate or triplicate.

Calculations, statistics, and analytical error. Standard formulas were used in calculating specific activities, K_e and TBW, including corrections for isotope excretion in the urine (21, 26). Whenever a 24 hour interval elapsed between K_e and TBW determinations, the K_e was corrected to the time of TBW measurement by metabolic balance for potassium by using measured intake and urinary losses. The plasma potassium and sodium concentrations, expressed in milliequivalents per liter of plasma water, were derived from the measured plasma sodium and potassium concentrations and plasma water content.

Conventional statistical equations were used to calculate standard deviations (S.D.) and correlation coefficients (r). The probability (p) of a correlation coefficient being obtained by chance was evaluated by the "t" test (27).

RESULTS

The data obtained in this study are listed in Tables II and III. There were 18 subjects with metabolic acidosis, 14 with metabolic alkalosis, 7 with respiratory alkalosis, 10 with respiratory acidosis, and 25 with normal acid-base status or without readily identifiable acid-base disturbance. The K_p varied from 1.93 to 6.68 mEq. per L. of plasma water, Na_p from 114.7 to 172.5 mEq. per L. of plasma water, Cl_p from 85.1 to 121.3 mEq. per L. of plasma, arterial blood pH from 7.15 to 7.59 units, venous blood pH from 7.11 to 7.51 and pCO_2 from 15.0 to 74.5 mm. Hg. The range of body composition values was also quite wide: TBW varied from 49.3 to 76.8 per cent of body weight; K_e ranged from 21.2 to 49.6 mEq. per Kg. of body weight and from 46.7 to 154.9 mEq. per Kg. of dry body weight.

TABLE IV

Relationship between plasma potassium concentration and arterial pH

pH range	Plasma potassium concentration mEq./L. plasma water	
	Mean	S.D.
7.14–7.36	5.10	±0.89
7.37–7.46	4.66	±0.50
7.47–7.60	3.58	±1.04

Plasma sodium concentration and plasma potassium concentration

The lowered intracellular potassium content frequently noted in such states as congestive heart failure is almost invariably associated with increased intracellular sodium concentration; this has been demonstrated in muscle (28–30) and in erythrocytes (31, 32). Hyponatremic and sodium retentive states have been reported to be associated with diminished renal excretion of potassium and titratable acid; this has been attributed to excessive proximal resorption of sodium, with consequent diminution in the amount of potassium secreted by exchange with sodium in the distal tubule (33). In dogs, however, the tendency to develop hyperkalemia in association with hyponatremia and sodium depletion could not be explained by renal retention of potassium (13). Conversely, administration of potassium to hyponatremic subjects has resulted in elevation of serum sodium levels (34), perhaps as a result of extrusion of sodium from potassium-depleted cells. The multiple determinants of the plasma sodium concentration and its apparent independence of extracellular pH, however, render unlikely any significant direct correlation between K_p and Na_p (15). The correlation between K_p and Na_p ($r = -0.22$) indicates that K_p tends to vary independently of Na_p or plasma osmolality. No significant difference in this correlation was found in the individual subgroups with either metabolic or respiratory acid-base disturbances (see Table V).

Plasma potassium concentration and extracellular pH

Numerous observations have confirmed the inverse relationship between the plasma potassium level and extracellular pH. The *in vitro* demonstration that acidification of the nutrient medium with mineral acid induced extracellular migration of potassium (35) led to experiments in nephrectomized animals subjected to acid and alkaline loads (36–39), artificial induction of respiratory acidosis and alkalosis (3–11) and infusion of acid and alkaline loads into humans (1). A direct re-

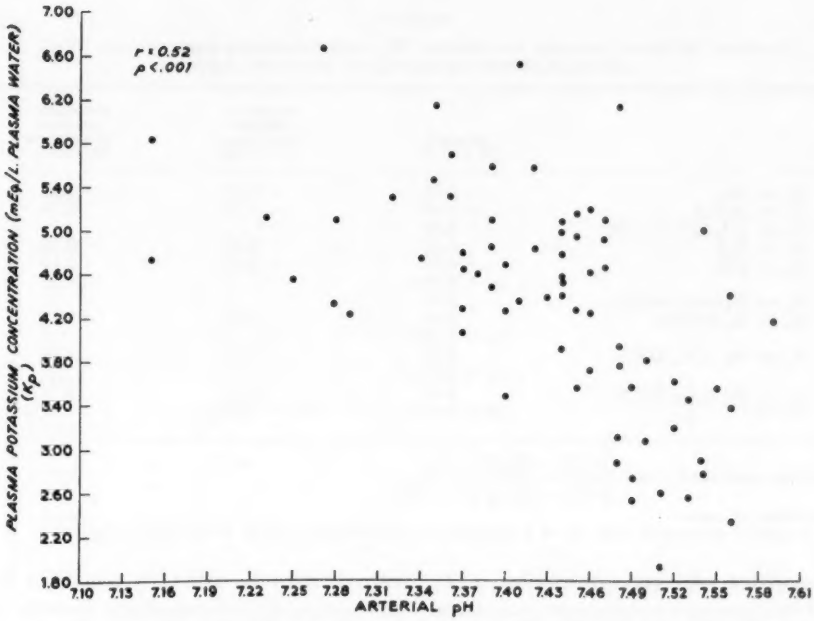

FIG. 1. RELATION BETWEEN PLASMA POTASSIUM CONCENTRATION AND ARTERIAL pH

lationship between hyperkalemia and acidosis and between hypokalemia and alkalosis without accompanying changes in body potassium content seems well established. The mean values for K_p according to pH_a subgroups, as summarized in Table IV, verify this trend. However, these are by no means invariable associations. Early respiratory alkalosis and the period immediately following respiratory acidosis have been reported to be associated with initial or further elevations of K_p, ascribed either to splanchnic release of potassium or to epinephrine effect (9, 10, 40, 41). Respiratory alterations of acid-base balance probably involve different buffering mechanisms than metabolic disturbances, *i.e.*, the buffer capacity of the extracellular fluids is usually increased with respiratory acidosis and decreased with metabolic acidosis, whereas the converse obtains in respiratory alkalosis and metabolic alkalosis. Furthermore, the nature of the metabolic disturbance seems to influence differentially the accompanying ionic redistributions (42, 43). Clinically, both alkalosis and acidosis often are not associated with significant deviations of plasma potassium level from the normal. These exceptions to the correspondence between K_p and extracellular pH attest to the importance of other regulatory factors. In this study the correlation between K_p and pH of extracellular fluid as represented by arterial blood was significant, $r = -0.52$ ($p < 0.001$) (see Figure 1 and Table V). If the cases are limited to those with $pH > 7.30$, then $r = -0.60$. A still higher correlation, $r = -0.64$, was found within the category of metabolic acid-base disturbances, while, surprisingly, in the respiratory subgroup, $r = -0.16$. No significant difference in correlation was found with venous blood pH. The influence of changes in pCO_2 on K_p proved to be negligible; for the entire group, there was no correlation at all, $r = -0.03$. In the metabolic disturbance subgroup a minor negative correlation was noted between K_p and pCO_2, $r = -0.36$; the respiratory subgroup demonstrated a similar degree of correlation in the opposite direction, $r = 0.30$.

Plasma potassium concentration and body content of potassium

Without doubt hypokalemia can be induced by loss of body potassium, if the loss is prolonged,

TABLE V

Correlation of plasma potassium concentration (K_p) with plasma sodium concentration (Na_p), plasma pCO_2 and total exchangeable potassium (K_e)*

	All cases N† = 51–74	Metabolic acid-base disturbances N = 26–32	Respiratory acid-base disturbances N = 11–17
K_p vs. Na_p	− 0.22	− 0.24	− 0.20
K_p vs. pCO_2‡	− 0.03	− 0.36	0.30
K_p vs. pH_a (pH > 7.30)	− 0.60		
K_p vs. H_a‡	0.52	0.64	0.16
K_p vs. pH_a	− 0.52 (p < 0.001)	− 0.64	− 0.16
K_p vs. K_e/body weight	0.22		
K_p vs. K_e/DBW‡	0.39 (p < 0.005)	0.45	− 0.28
K_p vs. $H_a \times K_e$/DBW	0.58 (p < 0.001)	0.70	0.20
K_p vs. $\frac{H_a \times K_e/DBW}{Na_p}$	0.62 (p < 0.001)	0.74 (p < 0.001)	0.24

* Correlation coefficient, $r = \frac{\Sigma[(X - \bar{X})(Y - \bar{Y})]}{\sqrt{\Sigma (X - \bar{X})^2 \Sigma (Y - \bar{Y})^2}}$

† N = number of cases.

‡ pCO_2 = partial pressure of CO_2; H_a = hydrogen ion concentration; DBW = dry body weight.

acute or associated with stress or alkalosis (12). An attempt has been made to quantitate such an isolated relationship (2). No consistent correspondence between K_p and body potassium content as reflected by K_e determinations, however, has been demonstrated (44). As expected, the correlation

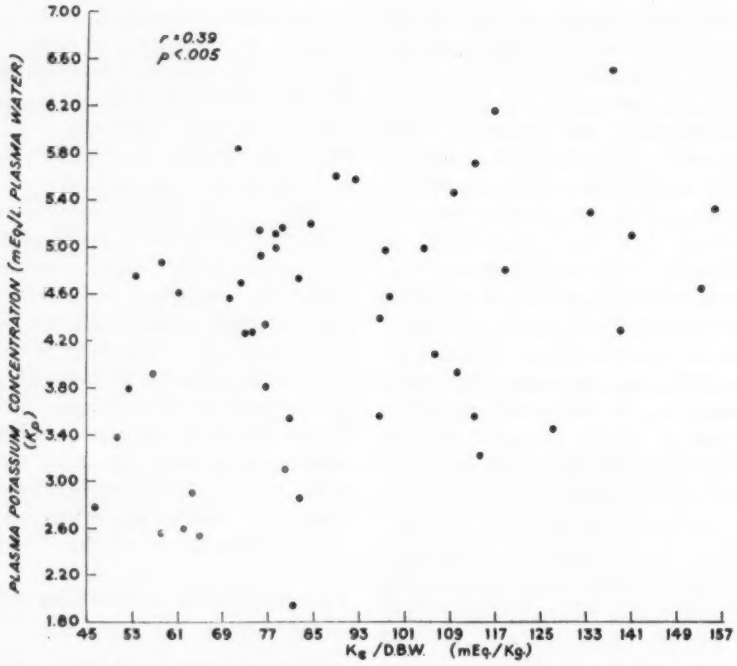

FIG. 2. RELATION BETWEEN PLASMA POTASSIUM CONCENTRATION (K_p) AND RATIO OF EXCHANGEABLE POTASSIUM TO DRY BODY WEIGHT (K_e/DBW)

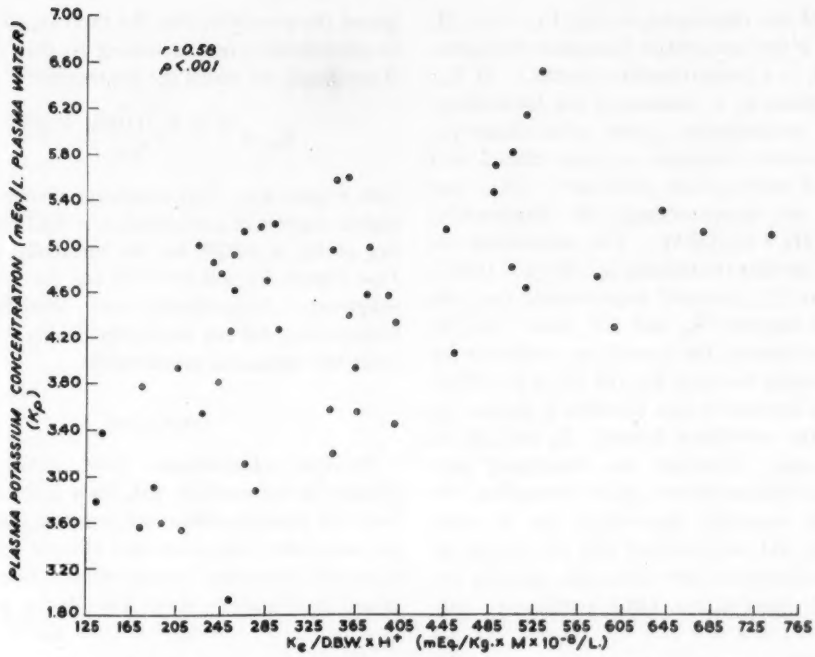

FIG. 3. RELATION BETWEEN PLASMA POTASSIUM CONCENTRATION (K_p) AND PRODUCT OF EXCHANGEABLE POTASSIUM PER KG. DRY BODY WEIGHT (K_e/DBW) AND ARTERIAL HYDROGEN ION CONCENTRATION (H^+)

found in this study between K_p and K_e/body weight was insignificant, $r = 0.22$ (Table V). One possible reason for this lack of correlation may be the difficulty in quantitating body potassium in the presence of the variable amounts of body fat and water in disease states. An attempt to circumvent this problem was made, first by deliberate selection of lean subjects only for study, and second by measurement of TBW, thus allowing calculation of the artificial parameter "dry" body weight (DBW). In fact, K_p did correlate significantly better with K_e/DBW ($r = 0.39$, $p < 0.005$) than with K_e/body weight (see Figure 2). In the subgroup with respiratory acid-base disturbances, $r = -0.28$ (see Table V). These imperfect correlations, while confirming the significant effect of alterations of body potassium content on K_p, attest to the need for a more comprehensive analysis of the factors affecting K_p, and again suggest that selective influences operate on K_p in metabolic and respiratory acid-base disturbances.

Plasma potassium concentration, body content of potassium, and extracellular pH

The almost invariable association of acid-base disturbances with those conditions which result in significant changes of body potassium content and K_p implies the existence of some overall quantitative relationship between K_p, body potassium content, and extracellular pH. Burnell, Scribner and co-workers (1, 2) have derived semiquantitative formulations which purport to define the relationships between changes in K_p and body potassium content and changes in K_p and extracellular pH. These formulations are of some value clinically, but provide no integrated account of the determinants of K_p at any one time. It seemed reasonable, therefore, to examine the proposition that the extracellular fluid pH, or H_a, quantitatively controls the gradient of potassium concentration across the cell membrane in the steady state, *i.e.*, in the absence of acute distorting factors such as changes in potassium balance. The simplest repre-

sentation of this relationship is: $K_p/K_{ic} = k \times H_a$ where K_{ic} is the intracellular potassium concentration and k is a proportionality constant. If K_e/DBW is taken as a measure of the intracellular potassium concentration (since extracellular potassium probably comprises no more than 3 to 4 per cent of exchangeable potassium) (45), then one may test experimentally the relationship: $K_p = k \times H_a \times K_e/DBW$. The correlation coefficient, r, for this relationship is 0.58 ($p < 0.001$) (see Figure 3), a modest improvement over the correlation between K_p and pH alone. In the metabolic subgroup, the correlation coefficient for the relationship between K_p and $H_a \times K_e/DBW$ is 0.70, an increase which parallels a similar increase in the correlation between K_p and pH in this subgroup. However, the respiratory subgroup again demonstrates a poor correlation, r = 0.20. The empirical observation that in some cases which did not correlate well the plasma sodium concentrations were abnormal, and the experimental observations cited previously, suggested the possibility that the ratio $H_a : Na_p$ might be more decisive in determining K_p than H_a alone. Accordingly we tested the expression:

$$K_p = \frac{k \times K_e/DBW \times H_a}{Na_p}$$

(see Figure 4). This maneuver resulted in still higher degrees of correlation, r = 0.62 for the entire group, r = 0.74 for the metabolic subgroup (see Figure 5) and r = 0.24 for the respiratory subgroup. Logarithmic and semilogarithmic formulations did not materially modify any of the described statistical relationships.

DISCUSSION

Previous observations have indicated that changes in extracellular pH, body potassium content, and plasma sodium concentration individually are associated with acute and chronic aberrations of plasma potassium concentration. The data obtained in this study show that plasma potassium

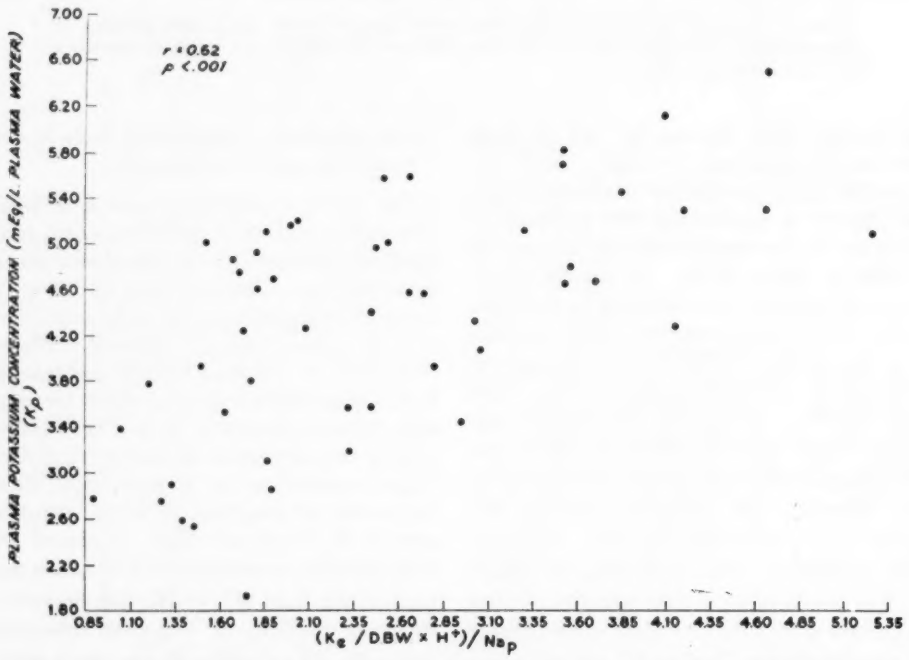

FIG. 4. RELATION BETWEEN PLASMA POTASSIUM CONCENTRATION (K_p) AND RATIO OF EXCHANGEABLE POTASSIUM PER KG. DRY BODY WEIGHT (K_e/DBW) × ARTERIAL HYDROGEN ION CONCENTRATION (H^+) TO PLASMA SODIUM CONCENTRATION (Na_p)

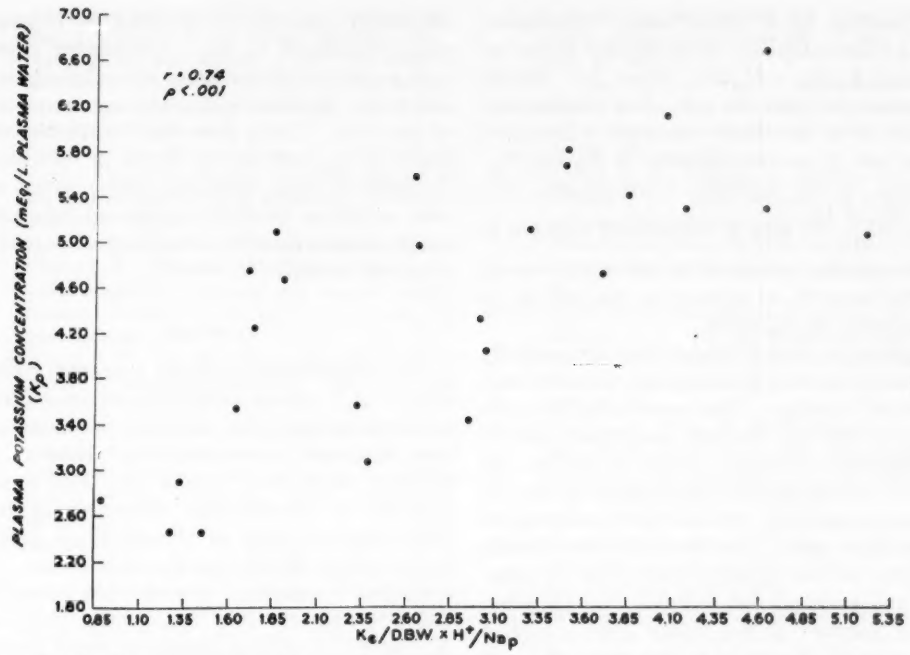

FIG. 5. RELATION BETWEEN PLASMA POTASSIUM CONCENTRATION (K_p) AND RATIO OF EXCHANGEABLE POTASSIUM PER KG. DRY BODY WEIGHT (K_e/DBW) × ARTERIAL HYDROGEN ION CONCENTRATION (H^+) TO PLASMA SODIUM CONCENTRATION (Na_p) IN PATIENTS WITH METABOLIC ACID-BASE DISTURBANCES

concentration is related to these three factors in decreasing order of importance. A summary of statistical correlations is presented in Table V. Striking differences are apparent between metabolic and respiratory acid-base disturbances, in that K_p seems much less dependent on these variables in the latter category (see Table V). A very close relationship ($r = 0.74$) has been found between K_p and the ratio $\frac{H_a \times K_e/DBW}{Na_p}$ in patients with metabolic acid-base disturbances.[2] It may be inferred from these observations that additional factors or different mechanisms affect K_p in patients with respiratory acid-base disturbances. This interesting finding suggests that H_a does not directly effect changes in K_p, but exerts its influence by its effects on intracellular buffering processes, and ultimately on intracellular hydrogen ion concentration. These effects probably depend on whether the primary disturbance is metabolic or respiratory in nature. Such a possibility is supported by Tobin's demonstration (43) that different degrees of acidosis and K_p change could be induced by equal loads of mineral and organic acids and by Fenn and Cobb's experiment showing that under certain conditions raising the extracellular concentration of dissolved CO_2 resulted in movement of potassium into cells (35). A possibly related factor may be the inherent lability of respiratory disturbances; the rapid diffusibility of CO_2 into and out of cells in response to changes in ventilation may preclude the establishment of the steady state envisioned in this study.

The proposal that K_p is proportional to the ratio $\frac{H_a \times K_e/DBW}{Na_p}$ may be interpreted as having fundamental physiological significance, provided that simplifying assumptions are valid. This relationship may indicate the passive distribution of potassium and hydrogen ions in proportion to resting transmembrane potential differences, prob-

[2] The consistently improved correlation coefficient resulting from the inclusion of Na_p in the denominator (although not statistically conclusive) strongly suggests that this observation is valid.

ably modified by a Gibbs-Donnan equilibrium. Such a relationship may be represented by the expression: $K_p/K_{ic} = H_a/H_{ic}$, where "ic" denotes concentrations inside the cell. The experimental verification of this simple construct is hampered by the lack of accurate measures of K_{ic} and H_{ic}. However, if the equation is rearranged, *viz.*, $K_p = \frac{K_{ic} \times H_a}{H_{ic}}$, then a resemblance emerges to the formulation presented in this study, except that the factor H_{ic} is replaced by Na_p and K_{ic} is approximated by K_e/DBW.

If potassium and hydrogen ions are passively transported across cell membranes, then the magnitude and direction of their movements, as conditioned by both the Donnan equilibrium and the transmembrane potential, would be similar. If, however, potassium is actively transported into the cell, it is conceivable that hydrogen ion competes for transport sites in the membrane in a fashion analogous to the apparent competition of potassium and hydrogen for secretion into the distal tubular segment of the kidney (46). Such a process would also reveal a dependence of K_p on H_a. A mechanism of this kind, however, would not in itself account for the differences noted in the correlations of K_p with pH_a between the group of patients with metabolic acid-base disturbances and the group with respiratory acid-base disturbances. The possible correspondence between Na_p and H_{ic} suggested above may arise from a dependency of intracellular osmolality on H_{ic} analogous to the dependency of extracellular osmolality on Na_p, which has been verified experimentally (15).

The expression K_e/DBW provides only a gross estimate of intracellular potassium content based on separate measurements of K_e, body weight, and total body water. Despite selection of lean subjects only, variable quantities of body fat were unquestionably present. Considering the close correlation between K_e and lean body mass it is probable that a measurably greater dependence of K_p on the product of H_a and body potassium content might be found if K_e were expressed in units of lean body mass (21, 47). The high incidence of hepatic disease may have been a complicating factor in this group of patients; the liver probably participates in changes of K_p during respiratory disturbances since the initial rise in K_p in early respiratory alkalosis is abolished in hepatectomized animals (9, 41, 48). The number of cases with respiratory disturbances was relatively small and it was not feasible to study any severe cases of this type. Finally there may be specific influences on K_p such as the effects of epinephrine secretion or other hormonal factors which may alter potassium gradients across cell membranes independent of the effect of hydrogen ion or body potassium content (40, 49–51).

SUMMARY

The relationships of plasma potassium concentration (K_p), plasma sodium concentration (Na_p), extracellular fluid pH, and body potassium content, expressed as exchangeable potassium per kilogram of dry body weight (K_e/DBW), were explored by simultaneous measurements in a heterogeneous group of chronically ill patients with a variety of acid-base disturbances.

Plasma potassium concentration correlates poorly or slightly with Na_p, plasma pCO_2 and K_e/DBW. A modest correlation exists between K_p and arterial blood hydrogen ion concentration (H_a) which is enhanced significantly when K_p is plotted against $\frac{H_a \times K_e/DBW}{Na_p}$. Striking differences in correlation appear between subgroups of patients with metabolic and respiratory acid-base disturbances. In the group of patients with metabolic disturbances a close correlation was found between K_p and $\frac{H_a \times K_e/DBW}{Na_p}$ which is not apparent in the group with respiratory acid-base disturbances.

The physiological implications of these findings are discussed, and possible differential mechanisms in these two types of acid-base disturbance are presented.

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HEPATIC METABOLISM OF PHENYLALANINE DURING DEVELOPMENT *

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The phenomenon of substrate induced enzyme formation has been amply documented in studies of microbial systems (1), and similar adaptive changes in enzymic activity in the tissues of vertebrate forms have been described by Knox, Mehler and Lin (2, 3) and by others (4, 5). The suggestion has been made (4, 6) that substrate induction may be a mechanism involved in those changes in enzymatic activities that normally occur during development and differentiation. An extension of this concept might predict that changes in the tissue level of a given substrate during development would be associated with alterations in the activities of enzymes concerned in the metabolism of that substrate. The present studies were initiated to test this hypothesis, using hepatic metabolism of the essential amino acid phenylalanine as a model system. Aside from incorporation into protein, which is common to all amino acids, the known reactions of phenylalanine in mammalian liver include only transamination to yield phenylpyruvate (7–9) and hydroxylation at the *para* position to form tyrosine (10). A previous report from this laboratory (11) established that the enzyme system forming tyrosine from phenylalanine is essentially absent during fetal life in the rat, developing to the level of the adult several days after birth. In the present report, this observation has been extended to other mammalian species and the nature of the enzymic deficiency in the liver of fetal animals has been determined. In addition, transamination of phenylalanine in rat liver has been studied, with particular reference to changes in activity of the enzyme during fetal and neonatal periods of development. A preliminary report concerning some of these studies has been published (12).

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METHODS AND MATERIALS

Tissue sources. The rats (Long-Evans) and rabbits used in these experiments were from laboratory colonies. The procedure for the determination of gestational age of rat fetuses has been described (13); the age of rabbit fetuses is estimated from the date of placing the male with the female. Pig fetuses 19 to 20 cm. long (crown to base of tail) were obtained at the abattoir. The sample of liver from a human premature infant was obtained at autopsy, two to three hours after death.

Enzyme preparations. Livers were removed and placed in cold saline or sucrose solutions as quickly as possible. Homogenates were prepared in 0.15 M KCl or 0.25 M sucrose using a Teflon homogenizer; livers of fetal and newborn (less than 10 days of age) litter mates were pooled. Preparation of mitochondria and other subcellular fractions from sucrose homogenates was carried out as described by Schneider (14) with some modifications (15). Fractions I and II, comprising the phenylalanine hydroxylase of rat liver, were prepared essentially as described by Mitoma (16).

Enzyme assays. Phenylalanine hydroxylase activity was assayed by measuring tyrosine formation, using the colorimetric procedure of Udenfriend and Cooper (17); details of additions to reaction mixtures are given in the appropriate tables. The high "blank" content of tyrosine in crude preparations was routinely corrected for by inclusion of samples in which enzymic activity had been initially terminated by trichloroacetic acid. Phenylalanine transamination was routinely assayed in the direction of phenylalanine formation by following the disappearance of phenylpyruvate, using a method suggested by Gilvarg (18). Small aliquots of trichloroacetic acid filtrates of reaction mixtures were pipetted into 1 cm. spectrophotometer cells, and sufficient NaOH was added to yield a concentration of 1.0 M in a final volume of 3.0 ml. The optical density of these mixtures was read at 321 mμ in the Beckman spectrophotometer, and the content of phenylpyruvate calculated from the extinction coefficient of the product formed under these conditions ($\epsilon = 1.45 \times 10^7$ cm.^{2} per Mole phenylpyruvate). Nonspecific absorption of reaction mixtures was found to be negligible, but it

TABLE I

*Effect of adult rat liver fractions on phenylalanine hydroxylase activity of liver preparations from fetal rats, rabbits, pigs and a human premature infant**

Experiment No.	Enzyme preparations added	μMoles tyrosine formed
1	Fetal rat liver soluble fraction	0.01
	Fetal rat liver soluble fraction + Fraction I	0.13
	Fetal rat liver soluble fraction + Fraction II	0.03
	Maternal rat liver soluble fraction	0.17
	Fractions I and II	0.07
2.	Fetal rabbit liver soluble fraction	0.07
	Fetal rabbit liver soluble fraction + Fraction I	0.34
	Fetal rabbit liver soluble fraction + Fraction II	0.04
	Maternal rabbit liver soluble fraction	0.43
	Fractions I and II	1.76
3	Fetal pig liver soluble fraction	0.01
	Fetal pig liver soluble fraction + Fraction I	0.62
	Fetal pig liver soluble fraction + Fraction II	0.02
	Fractions I and II	0.07
4	Premature human infant liver soluble fraction	0
	Premature human infant liver soluble fraction + Fraction I	0.73
	Premature human infant liver soluble fraction + Fraction II	0
	Fractions I and II	0.98

* All assays were carried out for 60 minutes at 35° C. Enzyme preparations were added to beakers containing 150 μMoles phosphate buffer at pH 6.8, 2 μMoles reduced diphosphopyridine nucleotide, 2 μMoles L-phenylalanine, and sufficient 0.15 M KCl to yield a final volume of 3.0 ml. Soluble fractions were prepared from 33 per cent homogenates in 0.15 M KCl by centrifugation at 105,000 × G for 60 minutes; 1.0 ml. of this preparation was used in each experiment. In each experiment either of the adult rat liver fractions alone formed only traces (less than 0.01 μMole) of tyrosine; assay of the two together in the amounts used is included in the table. Rat fetuses were at term (22 days) and rabbit fetuses were at 21 days of gestation; in these experiments assays of preparations of the maternal liver are included for comparison.

was necessary to include controls for nonenzymic removal of phenylpyruvate, apparently due to binding to acid-precipitated protein. The optical densities of these mixtures decreased slowly with time and were read at a fixed interval, usually two minutes, after mixing.

Reagents. Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), sodium phenylpyruvate, and trishydroxymethylaminomethane (Tris) were all products of the Sigma Chemical Company. Reduced DPN (DPNH) was prepared by reaction of DPN with ethanol and alcohol dehydrogenase as described by Rafter and Colowick (19). Crystalline lactic and alcohol dehydrogenases were from the Worthington Biochemical Corporation. Glucose-6-phosphate dehydrogenase was prepared from dried yeast according to Kornberg and Horecker (20). The phenylalanine hydroxylase cofactor (2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine) was a generous gift from Dr. Seymour Kaufman of the National Institutes of Health, Bethesda, Maryland.

RESULTS

Phenylalanine hydroxylation

It was first established by Mitoma (16) that the phenylalanine hydroxylase activity of rat liver can be dissociated by ammonium sulfate fractionation into two components. One of these (Fraction I) is found only in liver, is quite labile, and has been shown to be deficient in the livers of phenylketonuric individuals (21, 22). The second (Fraction II) is a stable enzyme which can be prepared from several tissues other than liver. Following the demonstration (11) that the livers of fetal and newborn rats are essentialy unable to form tyrosine from phenylalanine, experiments were carried out to determine whether this inactivity could be traced to deficiency in one, or in both, of the two enzymic components. Supplementing inactive soluble fractions of livers from fetal rats, rabbits and pigs with adult rat liver fractions shows quite conclusively that, in each case, the addition of Fraction I is sufficient to yield a combination with phenylalanine hydroxylase activity (Table I). A similar experiment was carried out using the soluble fraction of liver from a human premature infant, with the same result (Experiment 4, Table I). However, since this tissue was obtained at autopsy some time after the death of the infant, this result must be interpreted with caution, particularly in view of the apparent lability of this enzyme to autolysis (22).

Certain aspects of these experiments that were at first difficult to understand have been clarified by the studies of Kaufman (23, 24) which establish that this reaction requires a hitherto unrecognized cofactor. These aspects may be summarized as follows. *1*) While Fraction I loses activity rapidly on aging or dialysis when assayed with Fraction II, it was observed in several of these experiments that Fraction I preparations which had apparently lost most or all of their activity (when assayed with II) were still highly active in stimulating liver preparations from fetal animals (Experiments 1 and 3, Table I). *2*) As shown in Mitoma's initial report (16) and repeated in this laboratory, assay of the two rat liver fractions, varying one while holding the other constant, reveals important differences between them (Figure 1). If Fraction I is held at a constant level, and II varied, a typical enzyme concentration curve is obtained, while if II is constant and I varied, activity varies exponentially rather than linearly with the amount of I added. This suggests that Fraction I is providing more than one component to the system. *3*) As described in a preliminary report (12), the addition of varying amounts of Fraction I to inactive preparations of fetal liver results in activity which is essentially linearly related to the amount of I added; the multiple order response obtained when Fraction I is varied against II is not observed. These considerations can now be interpreted as indicating that Mitoma's Fraction I provides the rat liver system with the cofactor as well as with the specific hydroxylation enzyme, and that only one of these is required by fetal tissues. Thus the effect of Fraction I in stimulating inactive preparations from fetal animals could be due to deficiency of the fetal tissue in either of these components.

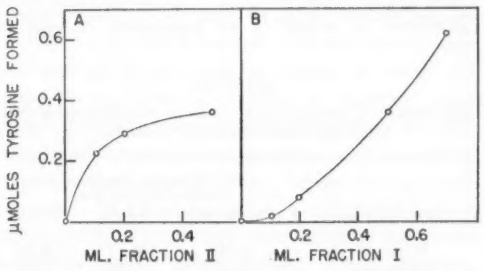

FIG. 1. EFFECT OF VARYING AMOUNTS OF RAT LIVER FRACTIONS IN HYDROXYLATION OF PHENYLALANINE

Reaction mixtures containing 150 μMoles phosphate buffer, pH 6.8, 4 μMoles L-phenylalanine, 1 μMole DPN, 10 μMoles sodium lactate, excess lactic dehydrogenase, rat liver fractions and 0.15 M KCl to 2.0 ml. were shaken at 35° C. for 30 minutes. After incubation trichloroacetic acid was added to five per cent, the mixtures were centrifuged and aliquots taken for tyrosine assay as described in "Methods." In A, all samples contained 0.5 ml. of Fraction I, and Fraction II was varied as indicated; in B, all contained 0.5 ml. of II, and I was varied as indicated.

TABLE II

*Specific requirement for hydroxylase (protein fraction I) by liver of fetal rats**

Additions	μMoles tyrosine formed
Fraction I	0
Fraction II	0.05
Fractions I and II	0.16
Fractions I and II + cofactor	0.91
Fetal rat liver soluble fraction	0.02
Fetal rat liver soluble fraction + Fraction I	0.27
Fetal rat liver soluble fraction + Fraction II	0.06
Fetal rat liver soluble fraction + cofactor	0

* The reaction mixtures contained 100 μMoles phosphate buffer at pH 6.8, 2 μMoles phenylalanine, 10 μMoles glucose-6-phosphate, 0.5 μMole triphosphopyridine nucleotide, 2 μMoles reduced diphosphopyridine nucleotide, excess yeast glucose-6-phosphate dehydrogenase, enzyme and cofactor additions as noted, and water to 1.5 ml. After 40 minutes at 37° C., trichloroacetic acid was added and the mixtures assayed for tyrosine as described in "Methods." The preparation of fetal rat liver was the 105,000 × G supernatant of a 33 per cent homogenate in 0.15 M KCl; 1.0 ml. of this preparation was used. Fractions I and II of adult liver were added in volumes containing 2.2 and 3.2 mg. of protein, respectively. Seventy-five μg. of the cofactor (2-amino-4-hydroxy-6, 7-dimethyltetrahydropteridine) was added where indicated.

A direct test of this problem was made possible by Kaufman's discovery that the reduced form of several pteridine derivatives can effectively substitute for the natural cofactor, although none of those yet tested is identical with it (25). Among the most active is 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, a sample of which was kindly made available to us by Dr. Kaufman. This cofactor was tested, along with aged protein fractions prepared from adult rat livers several days previously, for ability to stimulate hydroxylase activity of fetal rat liver. The results are summarized in Table II. It can be seen that this preparation of Fraction II is not completely free of I activity, and that I and II combined are relatively inactive unless supplemented with cofactor. This can be interpreted as indicating that I has

TABLE III

*Requirements for metabolism of phenylpyruvate by rat liver preparations**

Additions	μMoles phenylpyruvate metabolized
Homogenate	1.31
Mitochondria	0.21
Supernatant	0.27
Mitochondria + supernatant	1.18
Mitochondria + heated supernatant	1.22
Heated mitochondria + supernatant	0.15
Mitochondria + 5 μMoles L-glutamate	1.29

* Reaction mixtures containing 5 μMoles phenylpyruvate, 40 μMoles Tris buffer at pH 7.5, indicated additions and 0.25 M sucrose to 2.0 ml. were shaken at 37° C. for 30 minutes in the Dubnoff metabolic shaker, after which trichloroacetic acid was added and assays for phenylpyruvate carried out as described in "Methods." The mitochondria were washed three times with 0.25 M sucrose and the washings discarded; the supernatant fraction referred to is that part of the liver homogenate that is not sedimented at 8,500 × G. The amounts of each fraction added, in terms of equivalent weight of liver, were 0.5, 0.5 and 1.0 Gm. for homogenate, mitochondria and supernatant, respectively.

lost activity since its preparation, due to loss of the cofactor. Addition of II to the essentially inactive preparation from fetal rats stimulates to an extent attributable to the activity of II alone. Addition of the cofactor is without effect, while addition of I yields an active combination of even greater activity than I and II combined. This result establishes, then, that the fetal liver preparation contains both the cofactor and the enzyme of Fraction II, and is specifically deficient in the hydroxylase enzyme, represented by Fraction I.

Phenylalanine transamination

In preliminary studies of the routes of metabolism of phenylpyruvate, concentrated homogenates of rat liver were observed to metabolize this compound rapidly, either aerobically or anaerobically. On fractionation of homogenates by differential centrifugation, removal of phenylpyruvate could be shown to require both the mitochondria and the mitochondrial supernatant for maximal activity (Table III); the nuclear fraction (sedimented at 600 × G) was inactive. While the mitochondrial component is heat labile, the supernatant fraction lost no activity after boiling for five minutes and could be replaced by a variety of amino acids, of which glutamate was among the most active. These experiments could be repeated in an atmosphere of nitrogen without changing the results obtained.

The tentative conclusion that transamination was occurring was confirmed by paper chromatography of reaction mixtures; strong ninhydrin-positive spots corresponding to phenylalanine were obtained from each sample in which substantial amounts of phenylpyruvate had disappeared. A quantitative evaluation, measuring phenyalanine by the bacterial decarboxylase method of Udenfriend (26) was carried out, using washed mitochondria and glutamate under the conditions described in Table III. In duplicate samples, 1.83 and 1.87 μMoles phenylpyruvate disappeared and 1.75 and 1.78 μMoles phenylalanine were formed; in view of the uncertainties inherent in this phenylalanine assay these values were accepted as satisfactory stoichiometry.

The relative efficacy of several amino acids as amino donors in this reaction was studied in isolated subcellular fractions. Nuclear and microsomal fractions were essentially inactive with all the amino acids tested. Activity in mitochondrial and soluble fractions varied with each amino acid, but the distribution of activity in these fractions and the relative effectiveness of the amino acids were quite reproducible in several experiments. The data from a representative experiment are presented in Table IV. It is apparent that, when calculated in terms of an equivalent weight of

TABLE IV

*Effectiveness of various amino acids in transamination with phenylpyruvate in rat liver fractions**

Amino donor added	Phenylpyruvate disappeared (μMoles/hr./Gm. liver)		
	Mitochondria	Soluble	Total
None	0.36	0.12	0.48
Glutamate	6.24	2.53	8.77
Alanine	2.48	2.13	4.61
Aspartate	4.92	0.08	5.00
Glutamine	7.48	4.13	11.61
Leucine	3.12	1.13	4.25

* Reaction mixtures containing 50 μMoles Tris buffer at pH 7.5, 30 μg. pyridoxal phosphate, 5 μMoles phenylpyruvate, 10 μMoles of the L-isomer of each amino acid indicated, tissue preparation and 0.25 M sucrose to 2.0 ml. were shaken for 60 minutes at 37° C. under N_2. Mitochondria, washed three times, were added in an amount equivalent to 0.5 Gm. of liver, while the amount of soluble fraction (105,000 × G supernatant) added was equivalent to 0.3 Gm. of liver. Assays for phenylpyruvate were carried out after acid addition as described in "Methods."

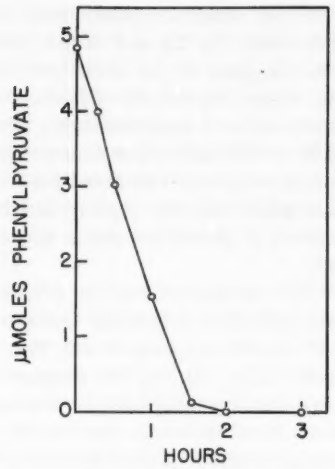

FIG. 2. TRANSAMINATION OF PHENYLPYRUVATE WITH L-GLUTAMINE IN RAT LIVER MITOCHONDRIA

The reaction mixture, containing 50 μMoles Tris buffer, pH 7.5, 60 μg. pyridoxal phosphate, 10 μMoles L-glutamine, 5 μMoles phenylpyruvate (4.84 μMoles by assay), three times washed mitochondria from 1 Gm. liver, and 0.25 M sucrose to 2.0 ml. was shaken at 37° C. under N_2 in the Dubnoff metabolic incubator. Small aliquots were taken at the times indicated and assayed for phenylpyruvate as described in "Methods."

liver, activity is rather evenly distributed between the two fractions. An exception is reaction with aspartic acid, which was in each experiment restricted to the mitochondria. These results suggest that there are probably several or many different transaminases capable of reaction with phenylpyruvate, although the possibility that some amino acids react indirectly, by prior formation of a specifically required donor, cannot be excluded. The rate of transamination with glutamate or glutamine is two to three times greater than with the other amino acids tested. Transamination with glutamine may be more rapid because this reaction goes to completion (Figure 2), probably due to coupling of transamination with deamidation of the α-ketoglutaramide formed (27). In contrast, reaction with glutamate tends toward equilibrium (Figure 3), as shown previously by Rowsell (9). It is of interest to note that the relative rates of this reversible reaction under the conditions used here (phenylalanine and phenylpyruvate at about 0.005 M, α-ketoglutarate and glutamate at 0.01 M) are quite different, the initial (first 30 minutes) rate in the direction of phenylalanine formation being approximately seven times greater than that of the reverse reaction. Rowsell found the two reactions to proceed at nearly equal rates, with all components added at 0.025 M. This disparity suggests a large difference in the Michaelis constant for the various reactants, with the phenylpyruvate-glutamate pair being bound much more tightly than phenylalanine and α-ketoglutarate.

Developmental study

The changes in activity of liver phenylpyruvate-glutamate transaminase that occur during late fetal and neonatal life in the rat are illustrated in Figure 4. Since this activity was shown to be distributed between mitochondrial and soluble fractions, the entire liver homogenates were assayed in this study. Under the conditions described in

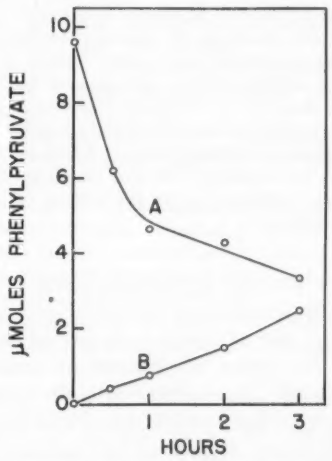

FIG. 3. APPROACH TO EQUILIBRIUM OF THE REVERSIBLE TRANSAMINATION BETWEEN PHENYLPYRUVATE AND L-GLUTAMATE IN RAT LIVER MITOCHONDRIA

Both reaction mixtures contained 50 μMoles Tris buffer, pH 7.5, 60 μg. pyridoxal phosphate, three times washed mitochondria from 1 Gm. liver, and sufficient 0.25 M sucrose to yield a final volume of 2.0 ml. In addition, 20 μMoles L-glutamate and 10 μMoles phenylpyruvate (9.6 μMoles by assay) were added to A, and 20 μMoles α-ketoglutarate and 10 μMoles L-phenylalanine were added to B. The mixtures were shaken at 37° C. under N_2; at the times indicated, appropriate aliquots were taken and assayed for phenylpyruvate as described in "Methods."

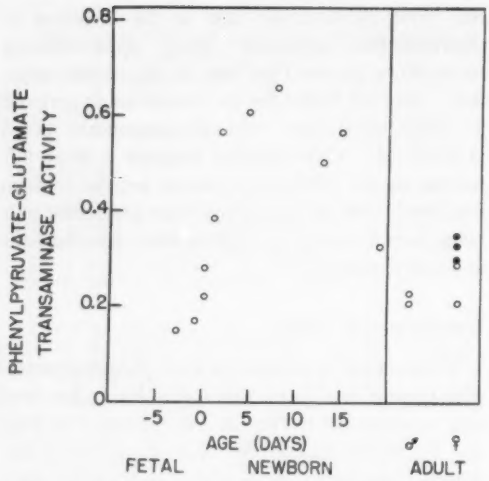

FIG. 4. CHANGES IN ACTIVITY OF HEPATIC PHENYLPYRUVATE-GLUTAMATE TRANSAMINASE DURING DEVELOPMENT OF THE RAT

Liver homogenates were shaken under N_2 for 30 minutes at 37° C. with 50 μMoles Tris buffer pH 7.5, 60 μg. pyridoxal phosphate, 10 μMoles phenylpyruvate and 20 μMoles L-glutamate in total volume of 2.0 ml. After acid addition aliquots were assayed for phenylpyruvate as described in "Methods"; activity is expressed in terms of μMoles phenylpyruvate disappearing per hour per milligram homogenate protein. Adults are here considered as rats weighing 100 Gm. or more. The solid circles are representative of the activity of livers of gravid females.

Figure 4 the rate of transamination is roughly linear with homogenate concentration over a 10-fold range, and all assays were carried out in this range. The choice of glutamate as amino donor was due to the observation that of the amino acids tested, it, together with glutamine, was consistently the most active. Glutamine was not used because the rate of reaction with this donor is apparently dependent on deamidation as well as transamination. In several of these experiments reaction mixtures were assayed for phenylalanine by the bacterial decarboxylase procedure, and in each case the amount of phenylalanine formed agreed to within 10 per cent of that of phenylpyruvate that had been metabolized.

Activity is present in the terminal days of fetal life, and by the time of birth is equivalent to that found in the livers of adult rats. During the neonatal period activity is high, reaching a peak during the first 10 days, which is approximately twice the usual adult level. By the end of the third week activity has dropped to the adult level, and, although not shown, the activity of livers of animals older than three weeks was consistently within this range (0.20 to 0.35 μMole per hour per mg. protein). Little difference was noted between male and female adults and the slightly increased activity in livers of gravid females is probably not significant.

While this manuscript was in preparation a report was published describing changes in the activity of several enzymes of rat liver during development (28). Among the enzymes studied was phenylalanine-α-ketoglutarate transaminase, which was assayed in soluble fractions by following the tautomerization of phenylpyruvate formed. While the findings reported differ in some details from those described here, the overall pattern of change, including development during late fetal life and high activity during the neonatal period, was similar to that seen in Figure 4.

DISCUSSION

Perhaps the most notable feature of the patterns of development of the two enzyme systems that are considered here is their lack of uniformity. Activity of phenylalanine hydroxylase has been shown to be deficient in late gestation, as well as for the first day or two of postnatal life in the rat (11). Although the complex kinetics involved in the study of this enzyme system make an accurate evaluation of the activity of crude preparations quite difficult, it was shown that activity is similar to that of adults by the third to twelfth day after birth. In contrast, the capacity to form phenylpyruvate from phenylalanine by transamination is present during late fetal life, and is equivalent to that of adults by the time of birth. A limited series of analyses in this laboratory has indicated that the free phenylalanine content of the livers of fetal rats of 17 to 21 days of gestation is at least as high as that of adults, when measured by the bacterial decarboxylase procedure in perchloric acid filtrates of homogenates. The methods used in these studies are of necessity crude, and can yield no information concerning what may be important intracellular or intercompartmental shifts of metabolite concentration.

Thus, while substrate induction may be involved in the mechanism of enzyme formation, these findings indicate that it is not simply the amount of substrate available that determines the time course of enzyme development. In this connection it might be noted that the development of tyrosine-α-ketoglutarate transaminase, an enzyme metabolically related to those considered here, has been shown to be under a measure of hormonal control (13).

The results reported here also establish that the characteristic inability of the livers of fetal mammals to form tyrosine from phenylalanine is due to absence or inactivity of the specific hydroxylase enzyme, as both the nonspecific enzyme and the cofactor are present. A recent report by Kaufman (25) has shown that biopsy specimens of liver from phenylketonuric individuals contain the cofactor as well as the nonspecific enzymic component of the hydroxylase system. Further study of the factors controlling the development of this enzyme in the newborn animal may prove to be of significance in this regard.

SUMMARY

Liver preparations from fetal rats, rabbits, pigs and a human premature infant were found to be essentially incapable of forming tyrosine from phenylalanine. Supplementing these inactive preparations with adult rat liver fractions indicates that each is deficient in the specific component of the phenylalanine hydroxylase system. The activity of fetal rat liver is not affected by addition of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, which is active as a cofactor in this system, indicating that inactivity is due to deficiency of the hydroxylase enzyme.

Phenylpyruvate undergoes transamination with several amino donors in the mitochondrial and soluble fractions of rat liver; glutamate and glutamine are particularly effective. In mitochondria, reaction with glutamine goes to completion, while reaction with glutamate approaches equilibrium. Assay of phenylpyruvate-glutamate transamination in the livers of fetal and neonatal rats indicates that activity is present during late fetal life, reaches the adult level by birth, and is higher than that of the adult during the neonatal period. Differences in the developmental patterns of phenylalanine hydroxylation and transamination are discussed from the point of view of substrate control.

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RENAL REABSORPTION OF BICARBONATE DURING ACUTE RESPIRATORY ALKALOSIS *

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It has long been recognized that acute respiratory alkalosis depresses the renal reabsorption of bicarbonate (1, 2), but the exact nature of the reabsorptive limit has not been defined. In the present studies, it has been demonstrated that acute reduction of plasma carbon dioxide tension depresses bicarbonate reabsorption to a rate which is essentially constant over a wide range of plasma bicarbonate concentrations. This pattern is analogous to that found in the normal but differs from that previously observed in acute respiratory acidosis, where reabsorption rises as a curvilinear function of plasma bicarbonate concentration (3).

MATERIALS AND METHODS

Twelve experiments were performed on female mongrel dogs anesthetized with sodium pentobarbital. Prior to each experiment, hydrochloric acid was infused in order to decrease the plasma bicarbonate concentration to roughly 10 mEq. per L. An endotracheal tube fitted with an inflatable balloon was introduced into the trachea and connected either to a calibrated volume displacement ventilator (Etsten Ventilator) or to a Mine Safety "Pneophore." Respiratory movements were inhibited by the administration of gallamine triethiodide (Flaxedil®) so that respiratory exchange could be controlled by the ventilator. Plasma pCO_2 was decreased to approximately 20 mm. Hg by hyperventilation while the dogs were breathing 100 per cent oxygen. When pCO_2 had been steadily maintained for at least one hour and adequate urine flow (2.2 to 5.8 ml. per minute) achieved by constant infusion of isotonic mannitol or isotonic saline solution, plasma bicarbonate concentration was progressively elevated by intravenous infusion of 0.142 to 0.535 M sodium bicarbonate to final values of approximately 35 to 45 mEq. per L. In most experiments it was necessary to increase ventilation slightly in order to prevent the rise in CO_2 tension that usually followed the rapid infusion of sodium bicarbonate. In most experiments there was a progressive reduction in blood pressure as blood pH approached 7.9 to 8.0. This hypotension was often accompanied by a drop in urine flow, as well as by hemoglobinemia and hemoglobinuria, and collection periods in which both hypotension and reduction in urine flow occurred were excluded from the study.

The clearance of exogenous creatinine was used as a measure of glomerular filtration rate. Urine was collected under mineral oil through an inlying catheter, and the bladder was emptied by manual compression at the beginning and at the end of each 10 minute collection period. Heparinized blood samples were drawn anaerobically from the femoral artery. Plasma and urine were analyzed for creatinine by a modification of the method of Bonsnes and Taussky (4) and for total CO_2 by the manometric method of Van Slyke. Blood and urine pH were measured anaerobically at 37° C. with a syringe type Cambridge Research Model pH meter. In a few experiments pH was measured at room temperature, using a factor of 0.01 unit per degrees C. for the correction to 37° C. Carbon dioxide tension in the blood and urine was calculated from the Henderson-Hasselbalch equation, using a pK′ for carbonic acid of 6.1 and a solubility factor equal to 0.0301 for plasma and 0.0309 for urine. Bicarbonate concentration was calculated as the difference between total CO_2 content and the dissolved carbon dioxide. Filtered bicarbonate was taken as the product of the filtration rate and the plasma bicarbonate concentration, corrected by a Donnan factor of 1.05.

RESULTS

Tables I through III show the data from three representative experiments. Plasma bicarbonate concentration was raised from initial levels of approximately 10 mEq. per L. to final values of 45, 39 and 35 mEq. per L., respectively. It is apparent that there was nearly complete reabsorption of bicarbonate during the initial periods when plasma bicarbonate concentration was 15 mEq. per L. or below. As plasma level was elevated to the range of 16 to 20 mEq. per L., frank bicarbonate excretion began and reabsorption promptly reached

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‡ Medical Research Fellow, National Research Council, Canada.

TABLE I

*Effect of acute sustained respiratory alkalosis on bicarbonate reabsorption during progressive elevation of plasma bicarbonate levels**

Time	Plasma		GFR*	Urine flow	HCO_3^-		
	pCO_2	HCO_3			Filtered	Excreted	Reabsorbed
min.	*mm. Hg*	*mEq./L.*	*ml./min.*	*ml./min.*	*mEq./min.*	*μEq./min.*	*mEq./100 ml. GF‡*
Dog No. 8, 11.0 Kg.							
−130	NaCl 0.154 M at 7 cc./min.						
−60	Dog breathing 100% O_2						
−2	$NaHCO_3$ 0.142 M at 7 cc./min.						
0–10	16	6.7	30.1	5.6	0.20	18	0.60
10–20	17	10.6	32.4	6.1	0.34	30	0.96
22	$NaHCO_3$ 0.240 M at 7 cc./min.						
25–35	17	13.3	34.2	6.1	0.46	37	1.24
35–45	17	16.9	36.7	6.3	0.62	60	1.53
47	$NaHCO_3$ 0.300 M at 7 cc./min.						
50–60	19	23.3	34.1	5.8	0.80	133	1.96
60–70	21	29.2	33.1	6.9	0.97	299	2.03
72	$NaHCO_3$ 0.350 M at 7 cc./min.						
75–85	22	33.5	29.0	7.4	0.97	396	1.98
85–95	22	36.9	30.7	8.4	1.13	547	1.90
97	$NaHCO_3$ 0.400 M at 7 cc./min.						
100–110	21	42.4	30.1	8.8	1.28	671	2.02
110–120	21	45.5	29.3	9.3	1.33	770	1.91

* Plasma bicarbonate values have been corrected by a Donnan factor of 1.05.
† Glomerular filtration rate.
‡ Glomerular filtrate.

a maximal constant rate of approximately 1.9 to 2.0 mEq. per 100 cc. of glomerular filtrate. In the three experiments pCO_2 ranged between 16 and 26 mm. Hg with an average value of approximately 20 mm. Hg.

The results of all 12 experiments are shown in Figure 1. On the abscissa is plasma bicarbonate concentration in milliequivalents per liter and on the ordinate bicarbonate reabsorption in mEq. per 100 cc., glomerular filtrate (GF). It can be seen that at plasma levels of 5 to 15 mEq. per L. there was nearly complete reabsorption of bicarbonate.

Frank excretion of bicarbonate usually began at plasma levels of 15 to 18 mEq. per L. At levels from 20 to 45 mEq. per L. reabsorption was virtually constant, averaging 1.9 mEq. per 100 cc. of glomerular filtrate. Variations in glomerular filtration rate during the course of the experiment were usually small and differed from the control values by no more than 20 per cent.

Plasma carbon dioxide tension for each experimental period is shown in the upper part of Figure 1. The extreme values for carbon dioxide tension were 13 and 26 mm. Hg, but the majority of the points lay between 17 and 22 mm. Hg.

DISCUSSION

The data indicate that during acute reduction of pCO_2 to approximately 20 mm. Hg, bicarbonate reabsorption is depressed to a rate which is independent of plasma bicarbonate concentration and which is essentially constant at an average value of 1.9 mEq. per 100 cc. of glomerular filtrate. In contrast, during acute respiratory acidosis the reabsorption of bicarbonate increases as a curvilinear function of plasma concentration (3), suggesting that the transport limits imposed by elevation and reduction of carbon dioxide tension are different in nature.

It is noteworthy that the reabsorptive pattern in acute respiratory alkalosis is similar to that of the normal, while the pattern in acute respiratory acidosis is analogous to that found with partial inhibition of carbonic anhydrase (Figure 2). The finding of two different reabsorptive patterns

TABLE II

*Effect of acute sustained respiratory alkalosis on bicarbonate reabsorption during progressive elevation of plasma bicarbonate levels**

Time	Plasma		GFR*	Urine flow	HCO_3^-		
	pCO_2	HCO_3			Filtered	Excreted	Reabsorbed
min.	*mm. Hg*	*mEq./L.*	*ml./min.*	*ml./min.*	*mEq./min.*	*μEq./min.*	*mEq./100 ml. GF*
Dog No. 12, 17.0 Kg.							
–95	Dog breathing 100% O_2						
–65	Mannitol 50 Gm./L. at 7 cc./min.						
–2	Mannitol 20 Gm./L., $NaHCO_3$ 0.166 M at 7 cc./min.						
0–10	25	11.6	62.6	3.2	0.73	1	1.16
10–20	25	12.3	66.7	2.7	0.82	1	1.23
20–30	24	12.9	67.7	2.2	0.87	1	1.28
30–40	24	13.5	61.0	1.8	0.82	1	1.34
42	$NaHCO_3$ 0.238 M at 7 cc./min.						
45–55	23	15.6	71.6	1.5	1.12	2	1.56
55–65	23	17.3	72.7	1.5	1.26	12	1.72
67	$NaHCO_3$ 0.300 M at 7 cc./min.						
70–80	24	19.7	72.3	1.5	1.42	38	1.91
80–90	24	21.5	86.6	1.9	1.86	120	2.01
92	$NaHCO_3$ 0.380 M at 7 cc./min.						
95–105	23	23.1	86.4	2.8	2.00	233	2.05
105–115	23	25.0	75.1	3.1	1.88	325	2.07
117	$NaHCO_3$ 0.452 M at 7 cc./min.						
120–130	23	26.9	77.5	4.0	2.09	469	2.09
130–140	23	28.8	71.3	5.1	2.05	621	2.00
142	$NaHCO_3$ 0.535 M at 7 cc./min.						
145–155	23	31.3	69.9	6.1	2.19	765	2.04
155–165	24	33.3	74.7	8.3	2.49	927	2.09
165–175	25	35.4	70.0	9.0	2.48	1,029	2.07
175–185	25	37.6	70.3	11.0	2.64	1,155	2.11
185–195	26	39.4	67.5	12.2	2.66	1,297	2.02

* See footnotes, Table I.

TABLE III

*Effect of acute sustained respiratory alkalosis on bicarbonate reabsorption during progressive elevation of plasma bicarbonate levels**

Time	Plasma		GFR*	Urine flow	HCO_3^-		
	pCO_2	HCO_3			Filtered	Excreted	Reabsorbed
min.	*mm. Hg*	*mEq./L.*	*ml./min.*	*ml./min.*	*mEq./min.*	*μEq./min.*	*mEq./100 ml. GF*
Dog No. 1, 16.4 Kg.							
–65	Dog breathing 100% O_2						
–30	Mannitol 50 Gm./L. at 7 cc./min.						
0–10	18	10.4	43.9	5.3	0.46	17	1.01
10–20	17	10.8	44.0	5.6	0.48	20	1.05
22	Mannitol 25 Gm./L., $NaHCO_3$ 0.166 M at 7 cc./min.						
40–50	19	14.6	43.6	5.9	0.64	39	1.38
50–60	17	16.1	49.3	6.4	0.79	55	1.49
62	$NaHCO_3$ 0.238 M at 7 cc./min.						
80–90	18	22.2	50.8	6.1	1.13	197	1.84
90–100	18	24.5	51.9	5.9	1.27	304	1.85
120	$NaHCO_3$ 0.300 M at 7 cc./min.						
120–130	21	33.0	47.3	6.0	1.56	635	1.96
130–140	21	35.2	44.5	5.8	1.57	711	1.93

* See footnotes, Table I.

might be accounted for if it is assumed, as has previously been suggested, that bicarbonate reabsorption takes place by means of a two step process involving a nonenzymatic and an enzymatic component, either of which can be rate-limiting (3, 5). According to this view, hydrogen ion secretion occurs as a first step in the reabsorptive mechanism and takes place independently of the enzymatic process within the tubular cell (3). A second step involving the enzyme, carbonic anhydrase, is concerned with the removal of hydroxyl ion released in the cell by the hydrogen secretory process. The specific role of the enzyme might be either to catalyze the reaction of $OH^- + CO_2 \overset{CA}{\rightleftarrows} HCO_3^-$, or alternatively to provide hydrogen ions for the neutralization of the OH^- ions by the reaction $H_2O + CO_2 \overset{CA}{\rightleftarrows} H_2CO_3$.

It has been suggested that the constant rate of reabsorption seen in the normal may be the consequence of a fixed maximal rate of hydrogen ion secretion which is determined by the carbon dioxide tension (perhaps through an effect on intracellular pH) (3). Thus, in the normal subject, this secretory step, rather than the enzymatic one, would be the slower of the two reactions leading to bicarbonate reabsorption and would be rate-limiting. The present study suggests that acute reduction in pCO_2 simply depresses the rate of hydrogen ion secretion and, therefore, of bicarbonate reabsorption to a new constant value. On the other hand, it has been postulated that, in acute respiratory acidosis or with partial inhibition of carbonic anhydrase, the rate of hydrogen ion secretion is high relative to enzyme activity and that the enzymatic step is rate-limiting. The evidence favoring

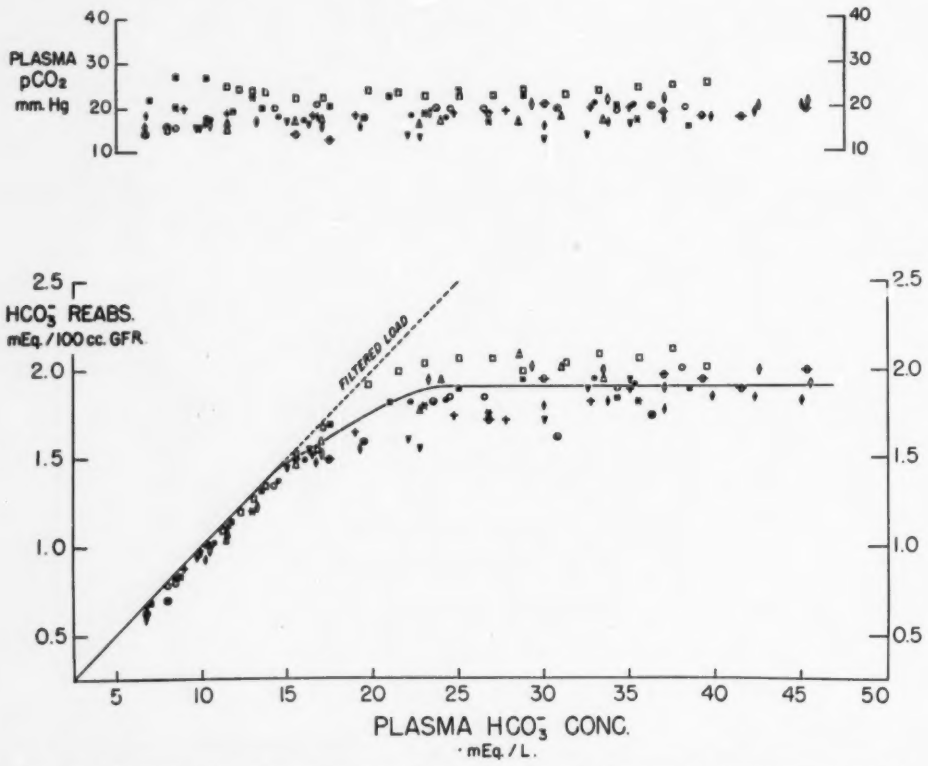

FIG. 1. EFFECT OF ACUTE SUSTAINED RESPIRATORY ALKALOSIS ON BICARBONATE REABSORPTION DURING PROGRESSIVE ELEVATION OF PLASMA BICARBONATE CONCENTRATION

The values for plasma bicarbonate have been corrected by a Donnan factor of 1.05.

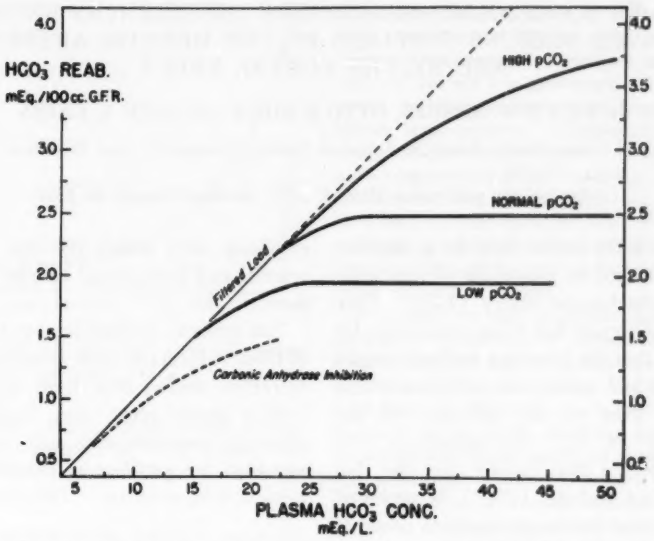

FIG. 2. DIAGRAM OF THE RELATIONSHIP BETWEEN PLASMA BICARBONATE CONCENTRATION AND REABSORPTIVE RATE WITH HIGH, NORMAL AND LOW PLASMA CARBON DIOXIDE TENSIONS AND WITH PARTIAL INHIBITION OF CARBONIC ANHYDRASE

this hypothesis has been discussed in detail elsewhere (3, 5). Regardless of these theoretical considerations, it seems clear that the pattern of the bicarbonate reabsorptive process may vary significantly under various experimental conditions.

SUMMARY

Renal reabsorption of bicarbonate was studied during acute, sustained respiratory alkalosis while plasma bicarbonate concentration was progressively elevated from approximately 10 mEq. per L. to final values ranging between 35 and 45 mEq. per L. With plasma carbon dioxide tensions of approximately 20 mm. Hg, reabsorption was depressed to an essentially constant rate averaging 1.9 mEq. per 100 cc. of glomerular filtrate. These data suggest that in the patient with acute respiratory alkalosis, as in the normal subject, carbon dioxide tension directly determines a fixed limit for bicarbonate reabsorption. This pattern contrasts with that seen in acute respiratory acidosis and with partial inhibition of carbonic anhydrase, where reabsorption rises as a curvilinear function of plasma bicarbonate concentration.

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STUDIES CONCERNING FUNCTIONAL DIFFERENCES BETWEEN LIVER REGIONS SUPPLIED BY THE HEPATIC ARTERY AND BY THE PORTAL VEIN *

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The small bile ducts in the liver of a number of species are enmeshed by a vascular plexus, supplied largely by the hepatic artery (1–3). This peribiliary arterial plexus has been considered by some as evidence that the bile duct epithelia might play an important and active role in the secretion of bile, possibly even to the exclusion of the parenchymal cells (4, 5). In support of this view, it was reported that in the dog the dye sulfobromophthalein sodium (BSP) is removed more completely, and for longer periods of time, when infused into the hepatic artery than when it reaches the liver by way of the portal vein. Since the vascular pathways traversed are common for both routes of injection, except for the intercalation of the peribiliary plexus in the path of part of the arterial blood, this was the site where the extra BSP uptake was thought to take place (6). By way of interpretation the possibility was suggested that BSP is preferentially excreted into bile when taken up by the tissues supplied by this plexus, *i.e.*, by the smaller bile ducts.

No direct test of this hypothesis was undertaken, however. With the availability of S^{35}-labeled BSP (7) the possibility suggested itself of using this material in conjunction with nonradioactive BSP to trace quantitatively two simultaneous BSP injections from two injection sites—portal vein and hepatic artery, for example—into bile, and thus to subject the concept of preferential biliary excretion of intra-arterially administered BSP to a critical test.

To validate the results of any such experiments requires some further analysis of the qualitative pattern of BSP excretion into bile and of the degree to which this might vary with the route by which the dye reaches the liver, as well as of the efficiency with which the dye is removed from arterial and from portal venous blood by the liver *in vivo*.

The present communication will deal with each of these subjects in turn, presenting validating bile secretion studies first, then the simultaneous injection experiments, and finally the extraction efficiency measurements and related studies dealing with the problem of obtaining representative hepatic vein samples.

METHODS

The experiments were carried out on female mongrel dogs, 9.5 to 15.0 Kg., which had been maintained in the laboratory on commercial canned dog food for at least six weeks. They were free of acute disease, and histopathological examination showed the livers of all animals used in these experiments to be free of significant abnormalities. Intravenous sodium pentobarbital anesthesia was employed throughout, respiratory rates being maintained between 14 and 20 per minute. Rectal temperatures were maintained above 35.5° C., if necessary, by the use of hot pads; in any one animal rectal temperature fluctuations did not exceed a range of 2.5° C.

Injections were made through polyethylene catheters, ending in 24 gage needles which were inserted into the gastrohepatic artery, 3 to 5 cm. proximal to the branching-off of the hilar hepatic arteries, and also into the portal vein, either just cephalad of the confluence of common intestinal and mesenteric veins, or at the junction of portal and gastrosplenic veins. These catheters were inserted at laparotomy, and brought to the body surface through the incision wound which was then closed by a single line of sutures. Catheter positions were rechecked at autopsy after the completion of each experiment. The possibility that dye injected via the hepatic artery might not reach the liver directly but be partially washed into the right gastric branches of the gastrohepatic artery was eliminated by careful placing of the arterial catheter and by an open loop placed around the gastric branch and tightened for ten seconds during and after the injections.

Blood samples were obtained from the femoral artery by direct puncture, and from the portal vein 3 cm. cephalad of the injection site through an additional polyethylene catheter. In addition, radiopaque catheters

* The opinions and assertions contained in this report are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

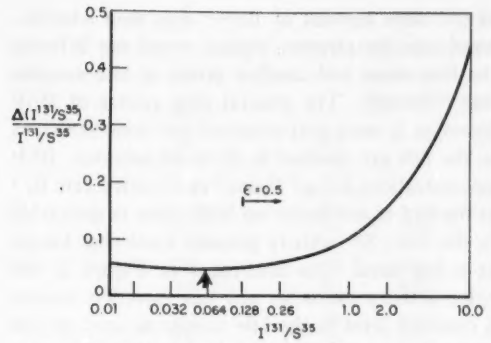

FIG. 1. ERROR IN I^{131}/S^{35} RATIO DUE TO CONSTANT COUNTING ERROR (± 3 PER CENT), AS A FUNCTION OF I^{131}/S^{35}

The interval marked $\epsilon = 0.5$ represents the interval covered by a typical experiment in which 50 per cent of the injected S^{35}BSP was removed from the blood in one passage through the liver.

were guided fluoroscopically to the following positions: *a*) vena cava, the tip being placed either 5 cm. cephalad, or 3 cm. caudad of its entry into the thorax above the dome of the diaphragm; *b*) hepatic vein, either one sampling catheter with its collecting tip 2 cm. cephalad of the point of wedging either in the right lateral lobe only, or two catheters with their tips placed in analagous positions in two hepatic veins in different lobes. Catheter sizes used were No. 10 in the vena cava, and No. 6 or (in the majority of experiments) No. 8 in the hepatic vein.

Blood samples from these catheters were withdrawn using a spring return plunger type of syringe equipped with directional ball valves (B-D Cornwall Pipetting Unit). This allowed collection of numerous 0.5 ml. samples at a rate of up to one sample per second from each catheter. The total dead space of each catheter together with its collection assembly was determined and found to be less than 1.5 ml. in every case. Mixing of samples in the system was negligible as shown by the sample concentration curves obtained on abruptly transferring the catheters from one to another solution of different composition, analyzing the catheter samples, and correcting for dead space lag.

Bile was collected into graduated centrifuge tubes from a plastic catheter inserted into the common bile duct after cholecystectomy. This catheter was placed at the same time as the injection catheters, brought out through the laparotomy wound, and placed so that the catheter opened into the collecting tubes at a level 2 cm. above the common duct.

Sodium phenoltetrabromphthalein disulfonate (BSP) was labeled with S^{35} in the sulfonic acid groups (7).[1] Colorimetric analyses for BSP in bile and blood plasma were carried out at 580 mμ. using the sample preparation and standardization methods previously described (8). S^{35} content was determined using air dried samples plated on tissue paper covered planchets in essentially infinite layer geometry in conjunction with a thin end-window Geiger-Müller detector. Standardization against known amounts of S^{35}BSP added to isotope-free samples of blood or bile, and details of the counting procedure have been reported elsewhere (8). All BSP analyses are recorded as milligrams of injected BSP equivalent to the optical density at 580 mμ. or to the amount of S^{35} detected in 1 ml. of sample.

P^{32}-labeled chromic phosphate colloid,[2] prepared according to the method of Dobson and Jones (9), was handled for injection or analysis using the precautions described by Brauer, Leong, McElroy and Holloway (10); blood radioactivity due to this substance was determined on air dried planchets, using an end-window Geiger-Müller counter and is expressed as percentage of injected dose per milliliter of sample.

I^{131}-labeled human serum albumin (IHSA), free of dialyzable radioactivity, was diluted for injection and analysis so that total protein concentrations did not fall below 1 per cent at any time. In these experiments IHSA was employed in combination with S^{35}BSP or with P^{32} $CrPO_4$ colloid as an internal standard to allow hepatic extraction efficiency determinations for these substances without obtaining unmixed representative hepatic vein blood samples. The two conditions underlying this use are: *a*) IHSA does not leave the vascular tree in amounts exceeding 1 per cent of the total injected during a single passage through the liver; this assumption has been tested and confirmed (11). *b*) The sole source of variable I^{131} and S^{35} or P^{32} content in the blood samples must be the injected isotope mixture; this condition requires elimination of recirculation and appropriate intervals between tests to allow removal of test substances from the blood stream. Both conditions are readily met (see Results, Figure 4).

Analysis of samples containing the isotope mixtures is designed primarily to determine S^{35}/I^{131} or P^{32}/I^{131} ratios. It entails radio-assay of two aliquots for each sample, using dry planchet and end-window detector (as described above for BSP and $CrPO_4$ assay) for one aliquot, and a wet 2 ml. sample in a well-type scintillation counter for the second. Since the ratios of response of the two detector systems are different for the γ-emitting I^{131} and for each of the two β emitters, the isotope ratio in an unknown containing the pair I^{131}–S^{35} or I^{131}–P^{32} can be computed from the ratio of the count rates on the two detectors for "unknown" and for the unmixed isotopes by the use of the appropriate simultaneous equations (for formula used see Appendix: *a*). Analysis of the solution of such a system of equations shows that the effect

[1] The authors are indebted to Dr. H. Brown Dunning, Jr., of Hynson, Westcott and Dunning, Inc., for making available the phenoltetrabromphthalein used in the synthesis of the labeled dye.

[2] The authors are indebted to Dr. E. L. Dobson, Donner Laboratory, University of California, Berkeley, Calif., for making available the $CrPO_4$ radiocolloid used in this work.

of uncertainties in count rate determination upon the calculated isotope ratio is a function of this ratio itself (see Appendix: *a*) as shown in Figure 1. For the system here employed the probable errors were ± 7 per cent for S^{35}/I^{131} and ± 15 per cent for P^{32}/I^{131} ratios in blood samples, and ± 2 and ± 4 per cent for the same ratios in the injection standards (see Appendix: *a*). Once these ratios are known in the appropriate blood samples and in the injection mass, the extraction efficiency, ϵ, can be calculated as the fraction of test substance removed from the blood in one passage through the liver using the relations:

$$\epsilon_{BSP} = 1 - \frac{S^{35}_{hep.\,v.}}{S^{35}_{aff.\,bl.}} = 1 - \frac{(S^{35}/I^{131})_{hep.\,v.}}{(S^{35}/I^{131})_{aff.\,bl.}}$$

where *hep.v.* refers to representative samples of hepatic venous blood, and *aff.bl.* refers to the corresponding afferent blood stream. If sampling is completed before recirculation takes place, $(S^{35}/I^{131})_{aff.bl.}$ is identical with $(S^{35}/I^{131})_{injected}$, and the final relations are:

$$\epsilon_{BSP} = 1 - \frac{(S^{35}/I^{131})_{hep.\,v.}}{(S^{35}/I^{131})_{injected}},$$

and a corresponding form for $CrPO_4$.

General plan of experiments. The following in brief is the plan of experimentation to be presented.

1. Experiments were performed to ascertain whether BSP reaching the liver by way of the hepatic artery is excreted into bile at a rate different from BSP introduced into the portal vein. These include: *a*) single injections of $BS^{35}P$ into either afferent vessel to determine whether the route of injection affects the composition or the specific activity of the complex of BSP derivatives recovered from bile; *b*) simultaneous injections of S^{35}-labeled BSP into one, and nonradioactive BSP into the other, afferent vessel followed by an appropriate period of bile collection, a second injection pair in which radioactive and nonradioactive injections are reversed and, finally, a second bile collection period.

2. Hepatic extraction efficiency for BSP delivered into either afferent vessel was determined. *a*) Attempts were made to accomplish this by hepatic vein catheterization. *b*) IHSA was used as an internal standard to allow analysis of the vena cava sample. This entails validation of the method and analysis of the catheterization artifact by the use of $CrPO_4$ colloid as test substance and application of the method to the BSP problem.

RESULTS

Single $BS^{35}P$ injections

About 0.5 mg. per Kg. was administered alternately into portal vein or hepatic artery of successive dogs, and bile collected in 15 minute portions until no further BSP color could be detected in two successive samples. A second injection of the same amount of $BS^{35}P$ was then administered into the afferent hepatic vessel not injected the first time, and another series of bile samples was collected. The general time course of BSP secretion is such that maximal dye concentrations in the bile are reached in 30 to 50 minutes; BSP concentrations fall off thereafter at such a rate that at the end of two hours no BSP color is detectable in the bile; S^{35} activity persists somewhat longer at a low level. As illustrated in Figure 2, the color/activity ratios for any one injection assume a constant level in the bile almost as soon as dye can be detected, and do not deviate from this value until 60 to 70 per cent of the injected S^{35} has been eliminated, and color due to the dye has become all but undetectable in the bile. The S^{35} excretion rate at this point has become very slow, less than 2 per cent of the peak value, and continues to decrease with a half-time of about one hour.

Table I summarizes the results of the series of successive injections. As previously observed in continuous infusion experiments (8), the ratio of S^{35}BSP to BSP color in bile varies considerably from dog to dog but appears to be quite constant for any one dog regardless of route of administra-

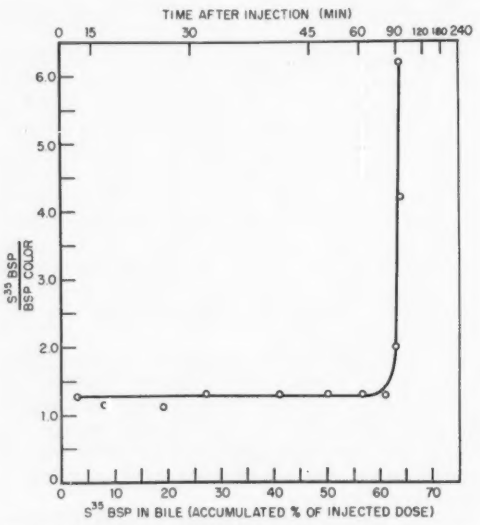

FIG. 2. COURSE OF S^{35}BSP/BSP COLOR RATIO IS A TYPICAL EXPERIMENT

Both the percentage of injected S^{35} accumulated in bile (bottom abscissa) and the duration of bile collection (top abscissa) are indicated (Dog 56-13).

TABLE I

Effect of route of injection on secretion of S^{35} into bile

Dog No.	Injection route and sequence	Peak of BSP* secretion into bile				Total bile		
		Bile flow	S^{35} BSP conc.	S^{35} BSP excretion rate	S^{35} BSP / BSP color	Collection period	S^{35} recovered	S^{35} BSP / BSP color
		µl./min.	*Gm./L.*	*µg./min.*		*min.*	%	
56–12	HA†	42	1.13	47.4	0.97	320	70.2	1.04
	PV	60	0.88	52.7	1.00	260	67.8	1.15
56–13	PV	53	0.93	49.4	1.17	230	79.8	1.34
	HA	63	0.80	52.4	1.26	240	81.7	1.47
56–14	HA	38	0.89	33.4	0.97	250	68.2	0.97
	PV	60	1.03	61.8	1.01	190	69.3	1.02
56–15	PV	36	1.15	40.5	1.13	215	74.6	1.27
	HA	45	0.96	43.2	1.26	230	79.5	1.43
Mean values	HA	48	0.95	44.1	1.12	256	74.7	1.25
	PV	50	0.99	48.3	1.10	253	72.3	1.22
Mean values	1st injection	42	1.13	42.8	1.07	264	73.2	1.16
	2nd injection	58	0.90	50.2	1.16	240	74.6	1.28

* Sulfobromophthalein sodium.
† HA = hepatic artery; PV = portal vein.

tion. On averaging separately the results after intraportal BSP administration and those after intra-arterial administration, comparison shows that route of injection does not affect the S^{35}BSP/BSP color ratios significantly, either in bile collected at the peak of BSP secretion, or in the accumulated total bile. The values are universally slightly higher for the accumulated bile samples because of the inclusion of some residual S^{35} excretion with little or no color equivalent. Absolute excretion rates are slightly higher for BSP injected into the portal vein than for that injected into the hepatic artery.

Differences between the group of first injections and the group of second injections are considerably larger than those between portal vein and hepatic artery injections. The rate of bile secretion itself is faster at the time of the second BSP peak than at the corresponding point after the first injection. This correlates with slightly higher rectal temperatures (+ 0.6°C. average) and respiratory rates (+ 20 per cent average). The peak BSP secretion rate is increased less than the bile flow rate. The S^{35}BSP/BSP color ratio appears to be slightly higher—about 8 per cent average for the peak of BSP secretion, 10 per cent for the accumulated bile—for the series of second injections than for the series of first injections.

Simultaneous injections

On the basis of these results, simultaneous injection experiments to establish the relative secretion rates of identical doses of BSP delivered via hepatic artery and via portal vein must provide for determination of specific activity/color ratios for excreted $BS^{35}P$ for each dog and, in addition, by alternation of injection routes between dogs, allow elimination of any small effects due to the order of injections. The design adopted to meet these requirements can be represented schematically as follows:

Dog No.	Injection pair No.	S^{35}BSP into	Nonradioactive BSP into
1	1	hepatic artery	portal vein
1	2	portal vein	hepatic artery
2	3	portal vein	hepatic artery
2	4	hepatic artery	portal vein

This scheme is to be replicated for successive pairs of dogs. The four individual doses of BSP making up the two injection pairs given to one dog were chemically equivalent; doses ranged from 0.30 to 0.37 mg. per Kg. for different dogs. After each first injection pair 180 minutes was allowed for completion of the dye excretion before the second injections were administered.

Figure 3 shows a typical set of results, and illustrates the method of deriving the activity/color

ratio for one animal. The basic graph represents rate of accumulation of S^{35} equivalent BSP as a function of accumulation of BSP color in the bile. In every case this plot yielded a straight line for each injection pair, with a slight upward hook near that point where BSP color excretion becomes vanishingly small while S^{35} excretion still continues at a slow rate (see Figure 2). Thus, "portal vein BSP" and "hepatic artery BSP" [8] are found to be excreted in constant proportion as long as BSP color excretion continues. If this proportion were known, the activity/color ratio in bile BSP could be determined directly from the slope of the curve for each injection pair; or conversely, if the color/activity ratio were known, the excretion rate ratio could be read off in the same fashion. Actually, however, both values are unknown, and a second complementary injection pair is needed to obtain the information necessary for further calculations. In terms of BSP color, the two injection pairs do not differ, and each value

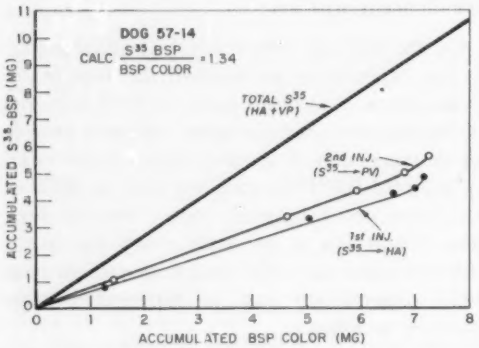
DOG 57-14
CALC $\frac{S^{35}\ BSP}{BSP\ COLOR}$ = 1.34
TOTAL S^{35} (HA + VP)
2nd INJ. (S^{35} → PV)
1st INJ. (S^{35} → HA)
ACCUMULATED S^{35}-BSP (MG)
ACCUMULATED BSP COLOR (MG)

FIG. 3. PLOT USED IN CALCULATION OF CHARACTERISTIC S^{35}BSP COLOR RATIO

Dog 57-14, a reciprocal paired injection experiment; see text for description.

[8] Actually, the distribution of "portal vein-injected BSP" in the liver is not exclusively to the hypothetical regions tributary to the portal vein because of incomplete uptake on the first passage and partial recirculation by way of the hepatic artery. A similar statement applies to "hepatic artery BSP." The actual relations can be calculated only after determination of single passage extraction efficiency (see below and Discussion). It is then found that "portal vein BSP" is actually 85 per cent portal vein and 15 per cent hepatic artery distributed and "hepatic artery BSP" is 65 per cent hepatic artery and 35 per cent portal vein distributed. With this usage restriction, however, the remainder of the argument can stand unaltered.

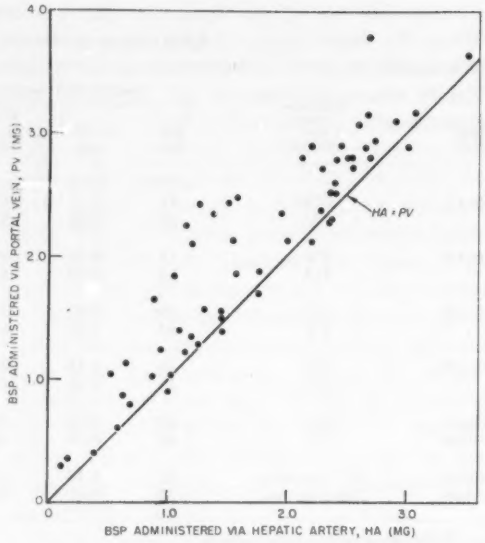

FIG. 4. ACCUMULATION OF PORTAL VEIN-DERIVED BSP AS A FUNCTION OF HEPATIC ARTERY-DERIVED BSP IN RECIPROCAL PAIRED INJECTION EXPERIMENTS

Diagonal HA = PV represents equal secretion rates.

of accumulated bile BSP color on the abscissa of Figure 3 defines corresponding stages of BSP excretion for either injection pair.

Since, however, the S^{35}BSP was injected first into one and then into the other afferent vessel, every number representing accumulated BSP color in the bile corresponds to two numbers, representing S^{35}BSP accumulated in bile after intraportal and intra-arterial S^{35} injection, respectively. Then, adding together the two S^{35}BSP values, corresponding to one BSP color value, yields the total amount of S^{35} which is equivalent to the total color excreted, and the ratio of these two values is the S^{35}BSP/BSP color ratio for the particular dog under study. The only assumption entailed in this calculation is that activity/color ratios must be independent of the route of injection; this point has already been established by the results shown in Table I. Thus, the slope of the summated S^{35}BSP line (HA + PV of Figure 3) is a true measure of the desired activity/color ratio.

Once this number is known for a particular dog, S^{35}BSP concentrations in bile can be converted to BSP color equivalents; by subtracting these from

the total BSP color one can calculate separately the contribution to the total bile BSP color made by each of the two injections constituting either injection pair.

The results of such calculations for a series of six dogs are presented in Table II which deals with the peak of BSP excretion, and in Figure 4 which traces the course of BSP elimination from its beginning until the rate of excretion dies away. In five out of six experiments, BSP injected into the portal vein accounted for a greater portion of the dye secreted into bile than an equivalent dose placed simultaneously in the hepatic artery; in the sixth experiment, the two doses were eliminated at substantially the same rate (Table II, last column). There is a difference between secretion rates at the time of the first and second excretion peaks; as before, bile flow and BSP secretion rates are slightly higher at the time of the second peak. If the experiments are divided into two groups, according to whether the $S^{35}BSP$ was first injected into the hepatic artery or into the portal vein, it is found that the factor of order of injections does not modify the conclusions; the ratio of portal vein BSP to hepatic artery BSP in bile at the time of peak secretion is 8 per cent lower for the "artery first" group, slightly less than would be expected on the basis of the S^{35}-BSP/BSP color ratio differences due to injection order found in Table I. However, even where this factor would tend to give erroneously low portal vein BSP/hepatic artery BSP ratios, the results still favor the portal vein by almost 20 per cent (Table II, bottom section, also Appendix: *b*).

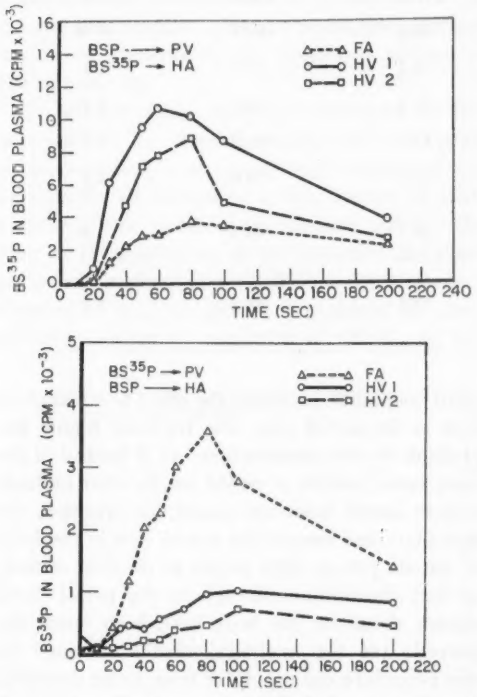

FIG. 5. DEMONSTRATION OF HEPATIC VEIN CATHETERIZATION ARTIFACT

Injection routes indicated for $BS^{35}P$ and for BSP; HA = hepatic artery, PV = portal vein. Ordinate represents relative $BS^{35}P$ concentrations in blood from the following: FA = femoral artery, HV = hepatic vein; the numbers 1 and 2 refer to catheter locations in right and in left lateral lobes, respectively. Multipore needles used for injections. (See text).

In Figure 4 the accumulation in bile of portal vein-injected BSP is plotted as a function of hepatic artery-injected BSP accumulation. Each injection pair is represented by four points approximately equally spaced between beginning and end of bile collection. The diagonal is the locus of points representing equality between the two routes of injection; points below this line imply preferential excretion of BSP injected into the artery, points above imply preferential excretion for BSP injected into the portal vein. The results clearly fail to indicate any special role of the hepatic artery in relation to BSP secretion; on the contrary, it appears that the portal vein distributes the dye to a region whence it is secreted somewhat more effectively than if it were distributed to the liver by way of the hepatic artery.

Extraction efficiencies

To establish whether such differences exist in dye removal from the blood as might prohibit the application of the biliary excretion results to a discussion of the postulated secretory function of the peribiliary arterial plexus, an attempt was made to estimate BSP extraction efficiencies by the use of hepatic vein catheterization. However, if rapidly collected series of blood samples were obtained not only from the hepatic vein catheters, but also from vena cava or femoral artery, results typified by Figure 5 were regularly obtained:

TABLE II

Effect of route of injection on secretion of BSP into bile; simultaneous injection experiments

Dog No., wt. in Kg. Dose: μg./Kg.	Route of S^{35} injected	Bile flow rate	BSP from hepatic artery	BSP from portal vein	Portal vein Hepatic artery BSP
		μl./min.	*μg./min.*	*μg./min.*	
56–3	PV	80	55.0	60.5	1.1
12.0					
0.33	HA	120	76.4	93.0	1.3
56–4	HA	60	22.2	44.5	2.0
9.1					
0.33	PV	110	31.8	58.3	1.7
56–11	PV	80	51.2	67.0	1.4
10.2					
0.37	HA	110	70.4	82.5	1.2
56–14	HA	90	30.5	32.2	1.1
12.8					
0.30	PV	140	46.0	50.2	1.1
56–15	PV	140	117.0	120.0	1.0
12.5					
0.34	HA	130	96.0	89.5	0.94
58–1	HA	180	44.2	46.5	1.1
10.0					
0.33	PV	160	39.2	46.3	1.2
Mean	1st injection	105	53.3	61.7	1.3
23.8					
0.33	2nd injection	129	60.9	70.0	1.2
Mean					
23.3	"Artery first"	123	35.7	46.3	1.3
0.34	dose				
Mean					
24.3	"Portal vein	110	77.7	85.4	1.2
0.32	first" dose				

if the S^{35}-labeled dye had been injected into the hepatic artery, one obtained the expected pattern in which the hepatic vein samples showed the earliest appearance of the dye and the highest BSP concentrations (Figure 5A). If the site of injection had been the portal vein, the hepatic vein catheter samples did not show high BSP concentrations until after the peripheral circulation samples, and even then hepatic vein BSP concentrations were less than peripheral ones, in a manner which would be expected if the BSP had reached the liver only by way of the peripheral circulation in the first place (Figure 5B).

This peculiar failure of the hepatic vein catheters to "see" portal vein injected BSP could be due either to a large portacaval bypass—unlikely in the normal dog—or to a circulatory adjustment within the liver, shunting blood away from the zone sampled by an intravenous catheter. A third possibility, ascribing the effect to streamlined flow in the portal vein, was rendered highly improbable by two observations: *a*) if instead of the open ended needle, a sealed needle with multiple minute lateral holes were used for injection, the injection mass entered the portal vein in the form of minute jets at right angles to the line of flow, so that distribution throughout the portal blood stream should at the least have been much improved; yet the analytical results obtained by this procedure did not differ from those described for the open needle injections. *b*) Seven experiments were carried out in which two catheters were introduced into hepatic veins of lobes to the right and to the left of the Cantlie-Sérégé plane, in addition to placing sampling catheters in the vena cava just above the diaphragm, and in the femoral artery. A total of nine injections of S^{35} BSP, and three injections of T1824 were adminis-

tered by the intraportal route in these animals. In every case the peak concentrations of dye attained in both hepatic veins were substanially *lower* than those found in the vena cava or femoral artery samples in the manner shown in Figure 5B; the time of appearance of the dyes in the several sites also differed significantly, the hepatic vein sample series being delayed by about 15 seconds relative to the femoral artery samples, and by about 24 seconds relative to the vena cava sample series. Similar anomalies were not observed with hepatic artery injections. It would have to be a rather extraordinary coincidence if in every one of these experiments streamlining of portal flow would be such as to bypass two sampling sites in the two liver moieties, unless one accepts (as we do) the conclusion that the presence of a catheter in the hepatic vein tends to minimize portal flow through the tissue elements in the immediate vicinity of the catheter tip.

To obviate these sampling difficulties, IHSA was used as an internal standard so that, prior to recirculation, vena cava blood samples could be used for determination of the fraction of BSP removed from blood in one passage through the liver by the application of the analytical and computational methods described (see Methods, and Appendix: *a*). A typical experiment is illustrated by Figure 6: a mixture of IHSA and test substance (S^{35}BSP in this case) is injected into the vessel chosen for study at an approximately constant rate over 15 seconds, Beginning at the same time as the injection, multiple sequential blood samples are drawn from the inferior vena cava just above the diaphragm, and are analyzed as described in Methods. The results, expressed as S^{35}/I^{131} ratios, are plotted in Figure 6.

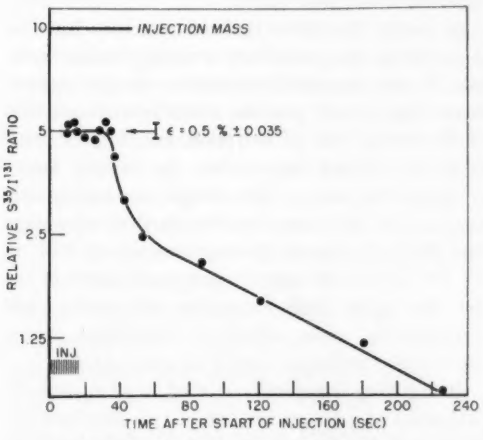

FIG. 6. INTERNAL TRACER METHOD

Graph used in calculation of extraction efficiency for BSP for portal vein-injected $BS^{35}P$ in Dog 57-25; see text for description.

TABLE III

Effect of route of injection on extraction of $CrPO_4$ from blood in one passage through the liver

Dog	Route of injection	$CrPO_4$ extracted in one passage
No.		%
57–4	HA	81.6
	PV	88.2
	PV	76.4
	HA	89.1
57–6	PV	52.7
	HA	57.4
57–8	HA	90.4
	PV	78.8
	PV	83.3
	HA	73.0
57–9	PV	68.0
	HA	79.2
	PV	78.5
Mean values	PV	78.5±13.1
	HA	75.5±11.2

The blood carrying the injected tracer mixture begins to arrive at the venous catheter six to eight seconds after the start of the injection. S^{35}/I^{131} ratios remain substantially constant from this point on until blood begins to arrive which has recirculated once more through the liver. This point is marked by a sharp decrease of S^{35}/I^{131} ratios which thenceforth continue to decrease in an approximately exponential fashion. The S^{35}/I^{131} ratio during the single passage plateau period is the value from which extraction efficiency of the liver for the test substance—BSP in this case—is calculated by comparison with the corresponding ratio in the injection mass.

Table III presents the results of a series of determinations, by this procedure, of $CrPO_4$ colloid extraction efficiency as a function of the route of injection. Although extraction efficiencies in general fall into the range of 70 to 90 per cent, it may be noted that an occasional dog may show consistently lower extraction efficiencies. The

TABLE IV

Effect of route of injection on extraction of BSP from blood in one passage through the liver

Dog	Route of injection	S^{35} extracted in one passage through the liver
No.		%
57–16	HA	58
	PV	51
57–22	PV	63
	HA	71
57–24	PV	46
	HA	49
	PV	46
57–25	HA	46
	PV	50
57–26	PV	36
	HA	36
	PV	35
Mean values	HA	52.0±8.1
	PV	47.2±4.2

route of $CrPO_4$ administration does not affect the efficiency with which this substance is removed from the blood stream in passage to the vena cava. Hence it may be concluded that a major vascular bypass between portal vein and vena cava or systemic circulation did not exist in these dogs.

The results of a series of measurements of BSP extraction efficiency are contained in Table IV. In three out of five dogs the hepatic arterial route of injection led to slightly higher efficiency of BSP extraction than the portal venous route; in one dog the reverse was seen; no differences were found in the fifth animal. These studies, therefore, lend some support to previous work indicating slightly greater BSP extraction from hepatic arterial blood (6). The difference is hardly significant, however, ($p > 0.1$), and in its application to the biliary secretion data would serve only to reinforce the conclusion that BSP, taken up by the those regions of the liver supplied with hepatic arterial blood primarily, is not excreted in preference to portal vein-distributed BSP.

DISCUSSION

In recent years, several problems connected with the functional anatomy of the liver have been revived by workers attempting to reconcile the results of morphologic and of physiologic experimentation. One of these problems has been raised by the description of the extensive vascular and lymphatic plexus enmeshing the bile ducts (1–3). The functional significance of this structure has given rise to a good deal of speculation concerning the possible secretory importance of the smaller bile ducts (12, 13). Since this plexus is supplied by the hepatic artery and represents the only vascular element not traversed to an equal extent by blood from portal vein and from hepatic artery, experimental approaches have dwelt on differences in the handling of substances reaching the liver by one or the other of these vascular routes.

BSP in particular has been examined from this point of view, and differences have been described in the persistence of the ability of the liver to remove this substance from the two afferent blood streams, the hepatic arterial route presumably showing satisfactory extraction for longer periods of time than the portal route (6). Unfortunately, at the time of these experiments it had not yet been recognized that BSP is not excreted as such in the bile (14) and that after prolonged BSP infusions the proportion of BSP proper in the blood stream is actually quite small (15, 16). Differences in BSP handling under these conditions are not readily interpretable, especially since it is clear that what ever other functions it may subserve, the peribiliary vascular plexus must be the site where considerable diffusional exchange between blood and bile constituents takes place (17).

In testing the thesis that an excretory function is served by the peribiliary arterial plexus, therefore, it now appeared essential to design experiments that would provide direct intercomparison of the actual rate of secretion into bile of BSP taken up as such from either the hepatic artery or the portal vein. This design, embodying bile analyses of necessity, has the further advantage that the high degree of concentration of BSP in the bile allows the use of such small doses of the dye that quite high extraction efficiencies—and consequently quite selective distribution along the vascular pathway—could be anticipated.

In applying isotopically-labeled BSP to such experiments as a tracer for one of two simultaneous streams of dye, its conversion to a whole family of optically indistinguishable dyes of varying color to radioactivity ratios poses a special problem (8). It was demonstrated in the present series of

experiments that the route of injection does not measurably affect this ratio, so that no bias is introduced into the analyses from this source. A possible small difference between successive injections in the same animal was recognized but could be eliminated by proper alternation of injection routes.

On this basis the key experiment of the present series is that represented by Table II and Figure 4, comparing the rates of secretion into bile of equal doses of BSP simultaneously reaching the liver of the same animal by way of either portal vein or hepatic artery. The experimental results do not under any circumstances admit of the interpretation that BSP injected into the hepatic artery is preferentially excreted. There is an indication, on the contrary, that the portal venous route affords a small but quite consistent advantage—BSP injected by this route being eliminated about 15 to 20 per cent faster at the time of peak BSP elimination.

The significance of this observation for the assessment of the functional role of the hepatic artery depends upon the additional information presented in Table IV concerning hepatic extraction efficiencies for BSP. The fact that these are either route independent, or that the hepatic arterial flow is somewhat more completely cleared of BSP—this in line with previous observations (6)—justified the conclusion that the vascular region supplied by the hepatic artery is not peculiarly active in transferring BSP from blood to bile. This would appear to remove any present justification from the postulate that the structures supplied by the peribiliary plexus (notably the smaller bile ducts) are endowed with any specific functional importance with regard to bile secretion. The present evidence does not, of course, bear upon a possible role of the structures in this region in the resorption of various components from bile in progress through the biliary tree.

Knowledge of the first passage extraction efficiencies for BSP allows an estimate of the actual distribution of BSP among vascular regions, provided one can estimate the relative blood flow rates through the two major vessels. A reasonable (and conservative) relation applicable to the present conditions would appear to be somewhere between 1/3 and 1/5 for the ratio hepatic arterial/portal venous flow (18–22); together with an initial extraction efficiency of 50 per cent this would lead to eventual distribution of BSP between the two vascular regions of 85/15 for the case of intraportal, and 35/65 for the case of intra-arterial injection. Thus, distribution differences would appear large enough to reveal any real differences in excretory activity.

There is not, at present, any explanation for the faster excretion into bile of portal vein-carried BSP suggested by Table II and Figure 4. One would be inclined to seek for explanations either in differences in mean oxygen tensions obtaining in regions receiving blood from the one or the other source, or in peculiarities of the sinusoidal circulation. An attempt was made to test this alternative by carrying out experiments analogous to the ones here reported under total oxygen tensions of 3.4 atmospheres resulting in "arterialization" of all circulating blood. The results suggested that oxygenation does not affect the excretory relations depicted in Figure 4; interpretation of these experiments, however, is rendered less than satisfactory by simultaneously occurring changes in respiratory movements and in rectal temperatures, both of which may have a bearing on bile flow and bile secretion.

Finally, attention may be called briefly to the results epitomized by Figure 5 and Table IV confirming in dogs under the experimental conditions of the present series the existence of a catheterization artifact as reported by Sapirstein and Reininger (23), and lending support to their general interpretation of the nature of this phenomenon. The present authors feel that a special reflex vasoconstriction is not required for interpretation of these observations but that the plasticity of blood flow distribution in the sinusoidal bed alone suffices to account for the deflection of portal flow away from a catheterized region. If borne out, this interpretation would assign to the hepatic arterial circulation a new role as an emergency supply to regions of the hepatic parenchyma, the circulation of which has been mildly compromised. Such a role could become of prime importance in the early stages of the development of liver injury by preventing the establishment of the vicious circle of liver disease envisioned by Himsworth (24).

As applied to hepatic vein sampling, the present results suggest that even if one encounters a catheterization artifact, in the normal liver at least, this is not important in BSP or $CrPO_4$ studies unless one specifically desires to discriminate between hepatic artery and portal vein as injection routes. Thus, if the test substances are injected into the peripheral circulation of such animals in such a way that they reach portal vein and hepatic artery in the same concentrations, the lack of difference between the efficiency of BSP or $CrPO_4$ removal from either blood stream implies that hepatic catheterization ought to yield meaningful results.

In the present case, of course, these assumptions could not be made *a priori* but, on the contrary, their verification was the object of the experiments. The use of an internal standard which allows sampling vena cava blood above the liver, in lieu of hepatic vein blood, was shown to obviate these difficulties and appears to be satisfactory for wider application whenever plasma protein flux across the hepatic tree is not excessive. It might be mentioned in passing that the limitations of this procedure are such that clear discrimination of first passage and recirculation phases becomes difficult unless samples are drawn close to the point of admixture of hepatic vein and vena cava blood streams.

The series of experiments here reported then, leads to the following conclusions. *1*) A special role of the peribiliary arterial plexus in the secretion of BSP into bile could not be demonstrated. It is suggested instead that this plexus and its adjacent structures contribute to the modification of bile composition by exchange of certain constituents among bile, lymph, and blood. *2*) Catheterization of the hepatic veins in the dog in these experiments led to changes in blood flow distribution which resulted in an excessive proportion of hepatic artery-derived blood in the catheter samples. It is suggested that this phenomenon may point to a hitherto unrecognized role of the hepatic artery as a safeguard against transient regional ischemia in the liver under mechanical stress. In its application to hepatic vein sampling this effect is not critical unless one desires to discriminate between the fate of a constituent reaching the liver by way of either the hepatic arterial or the portal venous blood stream. *3*) A method has been designed for overcoming this difficulty by the use of an internal standard in studies of extraction efficiency of test substances like $CrPO_4$ colloid, or BSP. *4*) Results of such measurements show no route-dependent differences in $CrPO_4$ extraction, and a (questionably) somewhat more complete extraction of BSP from the hepatic arterial stream.

SUMMARY

Differences in the handling of sulfobromophthalein sodium (BSP) reaching the liver have been claimed previously, depending upon whether administration was by way of the portal vein or the hepatic artery. This question has been investigated using S^{35}-labeled BSP to trace one of two equal simultaneous BSP doses (to hepatic artery and to portal vein) from injection site into the bile. The conditions under which such experiments can be interpreted have been elucidated. The results obtained indicate that route-determined differences in BSP secretion are either nonexistent or favor BSP reaching the liver in the portal venous blood stream.

The efficiency of BSP removal from portal vein and from hepatic artery blood has been studied using radioiodinated serum albumin as an internal standard to obviate the impossibility of obtaining representative hepatic vein blood samples by catheterization. BSP is extracted with an efficiency of 52 per cent from hepatic artery blood and 47 per cent from portal vein blood. From these two sets of results it is concluded that the hepatic artery, the peribiliary arterial plexus which it supplies, and the bile ducts enmeshed by this plexus do not play any special role in BSP secretion into bile.

In connection with the extraction efficiency studies, it was shown that blood samples from hepatic vein catheters contain an excessive proportion of blood originally supplied by the hepatic artery and that this is a genuine catheter artifact rather than the result of porta systemic shunts or of streamlined portal flow.

APPENDIX

a. Calculations bearing on the derivation of extraction efficiencies from double isotope injections incorporating an

internal standard. The general equations applicable to these calculations are:

1) $\frac{A}{B} = \frac{\beta_2 C_1 - \beta_1 C_2}{\alpha_1 C_2 - \alpha_2 C_1}$ for the isotope ratio,

and

2) $\frac{\Delta(A/B)}{A/B} = \left(K_I \frac{A}{B} + K_{II} + K_{III} \frac{B}{A}\right) \cdot \delta$ for the calculation of relative probable error in the isotope ratio. Here A and B are the relative amounts of isotopes A and B in arbitrarily chosen units (which, however, once chosen determine the numerical values of the remaining constants and of the results); numbers 1 and 2 refer to the two detectors employed, α_1 and β_1, and α_2 and β_2 are the count rates of one unit of pure A or pure B when placed under detector 1 or 2, and C_1 and C_2 are the count rates actually recorded for the experimental sample under detectors 1 and 2. $\Delta(A/B)$ is the error in A/B calculated to result from errors of $\pm 100\,\delta$ per cent in the determination of both C_1 and C_2; K_I, K_{II} and K_{III} are constants derived from appropriate manipulation of Equation 1:

$$K_I = \frac{1.41 \cdot \alpha_1 \alpha_2}{\alpha_1 \beta_2 - \alpha_2 \beta_1}$$

$$K_{II} = \frac{1.41\ (\alpha_1 \beta_2 + \alpha_2 \beta_1)}{\alpha_1 \beta_2 - \alpha_2 \beta_1}$$

$$K_{III} = \frac{1.41 \cdot \beta_1 \beta_2}{\alpha_1 \beta_2 - \alpha_2 \beta_1}.$$

Using as a unit of isotope an amount giving 1,000 cpm in a specified geometry on the end-window detector, the following are the numerical equations for the case of $I^{131} = A$ and $S^{35} = B$ and, for the detectors employed by the authors (1 referring to the scintillation detector, 2 to the end-window counter)

$$\frac{I^{131}}{S^{35}} = \frac{C_1 - 1.41 \times 10^{-3} C_2}{3.70\, C_2 - C_1}$$

and

$$\frac{\Delta(I^{131}/S^{35})}{I^{131}/S^{35}} = \pm \left(4.17 \frac{I^{131}}{S^{35}} + 4.17 + 0.017 \frac{S^{35}}{I^{131}}\right) \cdot 10^{-2}$$

where $100\,\delta = \pm 3$ per cent and it is assumed that the constants α_1 etc. are determined to within ± 1 per cent or better. The relation between I/S and the probable error is graphically represented in Figure 1. In practice, isotope mixtures for injection were prepared so as to give an initial I^{131}/S^{35} around 0.125; experience showed that in one passage through the liver this ratio was displaced to the left by an amount indicated approximately by the interval shown above the graph, corresponding to a BSP extraction efficiency of 50 per cent. The values for I^{131}/S^{35}, consequently, are reliable to ± 7 per cent or better throughout, provided the calculations are based on individual count rates reliable to ± 3 per cent as used for the blood samples. Injection standards were counted to $100\,\delta = \pm 1$ per cent. Similar relations hold for $CrPO_4$, but relative error here is ± 15 per cent for counting accuracy of ± 3 per cent.

b. Expressing all BSP values as "colorimetric BSP" (so that S^{35}BSP values are multiplied by the mean BSP color/activity ratio for the biliary BSP complex in the particular dog, yielding amounts of BSP or BSP complex equivalent colorimetrically to the recovered S^{35}), the ratio of artery-injected/portal vein-injected BSP is calculated from the following relations:

$$A = C - S\frac{\overline{C}}{\overline{S}}$$

$$V = S\frac{\overline{C}}{\overline{S}}$$

and

$$\frac{V}{A} = \frac{S\frac{\overline{C}}{\overline{S}}}{C - S\frac{\overline{C}}{\overline{S}}}$$

for the bile recovered after an injection pair in which the S^{35} went into the portal vein; and

$$A' = S'\frac{\overline{C}}{\overline{S}},$$

$$V' = C' - S'\frac{\overline{C}}{\overline{S}}$$

$$\frac{V'}{A'} = \frac{C' - S'\frac{\overline{C}}{\overline{S}}}{S'\frac{\overline{C}}{\overline{S}}}$$

for an injection pair involving S^{35} BSP injection into the hepatic artery. In these equations A and V, A′ and V′ are artery and vein-derived BSP after first and second injections; S and C, and S′ and C′ are corresponding S^{35}BSP and BSP color concentrations after first and second injections; and $\overline{S}$ and $\overline{C}$ are the cumulative S^{35}BSP and BSP color amounts (see Figure 3) from which the color/activity ratio is calculated. If the order of injections were as represented by the sequence of equations here, one would be dealing with a "portal vein first" experiment in the sense of Table II. The sequence effect illustrated at the bottom of Table I would lead to the prediction that S′ would be too large (relative to S) by about 10 per cent, and $\overline{S}$ would therefore be too large by about $\frac{10}{2}$ or 5 per cent. Examination of the equations for $\frac{V}{A}$ and $\frac{V'}{A'}$ shows that in this case both calculated ratios should be smaller than they would be in the absence of a sequence effect, and would deviate from the "correct" value by about the same amount (in the present example, provided A is not too different from V, and $\overline{S}$ from $\frac{\overline{C}}{2}$, the error would amount to about 10 per cent). By the same reasoning in "artery first" experiments the calculated values for $\frac{V}{A}$ and $\frac{V'}{A'}$ would be increased as a result of the injection sequence effect. As seen in Table II, the observed direction of the discrepancies between "portal vein first" and "artery first" experiments is as predicted, although the observed spread of mean values is somewhat smaller than would follow from these calculations.

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THE RELATION OF SULFUR METABOLISM TO ACID–BASE BALANCE AND ELECTROLYTE EXCRETION: THE EFFECTS OF DL–METHIONINE IN NORMAL MAN *†

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It has been known for many years that most of the inorganic sulfate in the urine is derived from the oxidation of sulfur-containing organic compounds. In the classical calculation of the "acid-base balance" of food each Mole of sulfur in the ash is assigned an acid equivalence of two, thus assuming its quantitative conversion to sulfuric acid or to some other strong acid or acids which yield a total of two equivalents of hydrogen ion per Mole of sulfur (1).

Until recently, however, there have been virtually no studies of the relationship of sulfur metabolism to acid-base balance. In 1956 Hunt (2) reported that the feeding of methionine or sulfur-containing foods to man increased the excretion of acid in the urine. He suggested that it is chiefly the oxidation of dietary sulfur that determines the urinary excretion of acid. Interest in the relation of sulfur metabolism to the acidification of the urine has also been stimulated by the observation that administration of methionine is an effective technique for maintaining an acid urine in some patients with chronic urinary tract infection (3). Small reductions in plasma CO_2 content have been observed in subjects given large doses of methionine (4), thus further suggesting a connection between the metabolism of organic sulfur and acid production.

Although on theoretical grounds the release of two hydrogen ions would appear to be an obligatory consequence of the biological oxidation of methionine-sulfur to sulfate in an aqueous medium, there is at present no experimental demonstration of this relationship. No detailed or quantitative information about the interrelationships of changes in systemic acid-base balance, urinary excretion of acid and electrolytes and the metabolism of organic sulfur is currently available. The present work was therefore designed to clarify this problem by carrying out balance studies of the effects of large loads of DL-methionine in normal men.

METHODS AND MATERIALS

Five balance studies were performed on three healthy young adult males who carried on their usual daily activities during the period of observation. Each study was preceded by a two to four day period of adaptation to the standard diet. The balance then began with a control period, followed by a period of DL-methionine loading, and a recovery period, each lasting five to six days.

Liquid formula diets were utilized, which provided 35 to 40 calories per Kg. body weight and 70 to 90 Gm. protein per day. A soybean flour [1] was used as the protein source, and had the following composition:

Protein	50 per cent
Fat	1 to 2 per cent
Carbohydrate	30 to 35 per cent
Calcium	0.2 to 0.4 per cent
Phosphorus	0.5 to 0.7 per cent
Potassium	57 mEq. per 100 Gm.
Sodium	< 1 mEq. per 100 Gm.
Chloride	< 1 mEq. per 100 Gm.

Corn oil and glucose served as sources of fat and carbohydrate, respectively. In three balance studies 200 mEq. sodium chloride was provided daily in the diet, while in two studies no sodium chloride was added. The subjects drank a constant amount of tap water and took one multiple vitamin capsule each day. Some subjects took small amounts of instant coffee either mixed with the diet or in the supplementary water, and in these cases the coffee was included in the diet analyses.

Since the soybean flour contained only 0.7 Gm. methio-

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[1] "Toasted Soy Protein" granular flour, kindly made available by Soybean Division, General Mills, Inc., Minneapolis, Minn.

TABLE I

Experimental data

Subject J. L. Normal NaCl intake

Day	Body weight	Blood pH	Serum				Urine															Stool				
			Total CO_2	Na	K	Cl	Vol.	pH	Total CO_2	T.A.†	NH_4	"Net acid"‡	Inorg. SO_4	Ethereal SO_4	Na	K	Ca	Cl	P	N	Total org. acids	Na	K	Cl	N	P
	Kg.		*mMoles/L.*	*mEq./L.*			*ml./day*		*mMoles/day*				*mEq./day*						*mMoles/day*	*Gm./day*	*mEq./day*	*mEq./day*			*Gm./day*	*mMoles/day*
1	65.64	7.38	29.1	143	4.3	108	1,575	6.56	29	5	37	21	36		185	62		195	9	9.0	46	5.3	19	1.6	1.9	31
2	65.71						2,024	6.96	39	4	28	−2	35		242	73		237	8	9.8	45	5.3	19	1.6	1.9	31
3	65.29						1,970	6.63	25	7	36	24	33		211	62		221	9	9.8	47	5.3	19	1.6	1.9	31
4	65.16	7.41	30.2	144	4.4	105	1,345	6.54	16	8	29	26	38		163	53	2.2	156	12	9.9	43	5.3	19	1.6	1.9	31
5	65.29						2,270	6.86	39	7	30	4	39	2.7	257	76		241	13	10.9	43	5.3	19	1.6	1.9	31
6	64.77						1,811	6.77	32	7	30	11	40	3.1	199	70	1.8	190	11	10.7	45	5.3	19	1.6	1.9	31
7*	64.77	7.40	30.2	144	4.4	104	2,121	5.76	6	17	65	81	143	4.0	221	60		197	13	13.2	53	4.3	19	1.6	1.7	25
8*	64.24	7.38	27.9	143	4.4	106	1,776	5.17		23	88	101	164		204	72	5.5	185	12	12.8	46	4.3	19	1.6	1.7	25
9*	64.08	7.37	27.0	142	4.4	104	1,696	5.18		22	99	121	166		184	71		181	9	12.7	50	4.3	19	1.6	1.7	25
10*	63.87						1,984	5.15		23	106	129	165		215	70	6.7	222	9	12.3	53	4.3	19	1.6	1.7	25
11*	63.87	7.38	26.2	143	4.3	106	1,924	5.13		27	114	141	171	1.0	192	79	5.0	200	14	12.1	55	4.3	19	1.6	1.7	25
12*	63.50						1,867	5.17		27	111	138	173	6.0	192	82		196	14	12.4	53	4.3	19	1.6	1.7	25
13	63.58	7.38	26.3	143	4.3	105	1,628	5.24		27	107	134	111		142	82	5.2	177	16	11.3	46	2.8	18	1.0	1.5	24
14	63.76						1,102	5.53	2	22	114	135	44		103	67	2.1	153	16	9.8	42	2.8	18	1.0	1.5	24
15	64.40	7.38	30.8	142	4.3	106	1,610	6.58	13	9	36	35	34		164	59		175	10	10.5	43	2.8	18	1.0	1.5	24
16	64.54	7.40	31.2	141	4.1	104	1,950	6.80	30	5	29	9	38	1.6	201	65	2.5	201	9	9.8	48	2.8	18	1.0	1.5	24
17	64.44	7.42	30.6	143	4.1	105	1,839	6.75	28	7	27	12	36		195	70		197	8	9.4	41	2.8	18	1.0	1.5	24
18	64.00	7.39	31.1	145	4.2	105	1,691	6.86	29	7	25	8	33	3.6	202	63	2.5	198	10	10.0	39	2.8	18	1.0	1.5	24
19	64.07	7.41	30.1	144	4.3	104																				

Basal intake: K 81 mEq. per day, Na 202 mEq. per day, Cl 202 mEq. per day, P 30 mMoles per day, and N 11.6 Gm. per day.

* DL-Methionine 13.9 Gm. per day, add ¼ to basal intake.

† Titratable acidity.

‡ $NH_4^+ + T.A. - HCO_3^-$.

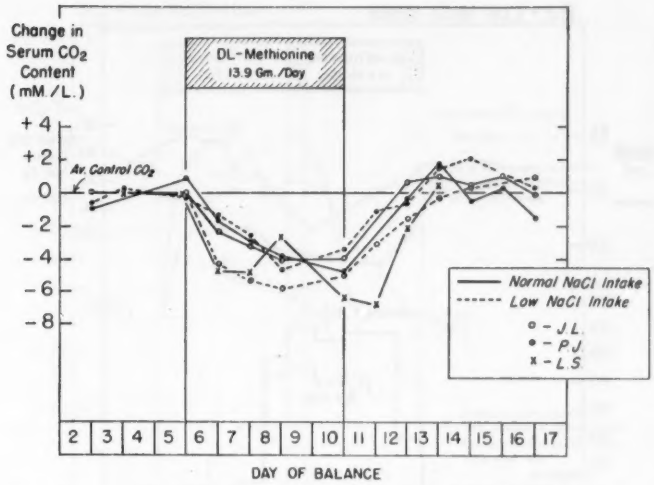

FIG. 1. INFLUENCE OF METHIONINE FEEDING ON SERUM CO_2 CONTENT IN FIVE EXPERIMENTS ON THREE NORMAL SUBJECTS

nine per 100 Gm., a supplement of 1.0 Gm. DL-methionine per day was provided in the basic diet in order to meet minimum daily requirements. An additional 13.9 Gm. DL-methionine per day was added to the basic diet during the period of methionine loading.

Urine was collected under mineral oil using thymol and phenyl mercuric nitrate as preservatives.

Diets were analyzed for total nitrogen, sodium, potassium, chloride and phosphorus. The methods for these determinations, as well as those employed for the analyses of blood, urine and stool have been previously reported (5) with the following exceptions: urinary ammonia was determined by Conway's micro-diffusion method (6), ethereal sulfates in urine were determined gravimetrically as barium sulfate (7) and chloride in serum, urine, stools, and diet was determined by a potentiometric titration (8). Urine pCO_2 and bicarbonate were calculated from the total CO_2 content and pH of the urine. Net urinary excretion of acid (referred to as "net acid") was calculated as the sum of ammonia plus titratable acid minus bicarbonate.

RESULTS

Analytical data from a representative balance on one subject (J. L.) are presented in Table I, and some of the pertinent results from the other balances are shown in Figures 1 through 4.

I. Effects of methionine loading

In each of the balance studies the administration of DL-methionine produced an immediate though modest drop in serum CO_2 content (Figure 1), but venous pH did not change. There were no detectable changes in the serum concentrations of sodium or chloride. Potassium concentration decreased by 0.3 and 0.7 mEq. per L. in the two balances without added salt.

Excretion of inorganic sulfate increased rapidly from control levels of 33 to 49 mEq. per day up to 150 to 179 mEq. per day by the second or third day of methionine administration. The maximum daily increments above the average control rates ranged from 110 to 130 mEq. per day. Excretion of ethereal sulfate was measured in two balances and did not change (Table I).

Associated with the sulfate diuresis was a prompt fall in the pH of the 24 hour urine collections to the range of 4.7 to 5.2 (starting from control values of 5.8 to 7.0). The pH of individual freshly voided specimens varied from 4.5 to 5.5 throughout the day. There was also an immediate and progressive rise in ammonium excretion starting from control rates of 19 to 37 mEq. per day and rising gradually to levels of 92 to 115 mEq. per day by the fourth or fifth day of loading. Titratable acid increased by an average of 15 to 22 mEq. per day and remained fairly constant throughout the period of loading. The excretion of "net acid" rose gradually and then leveled off after two or three days. As shown in Figures 2 through 4, the increments in "net

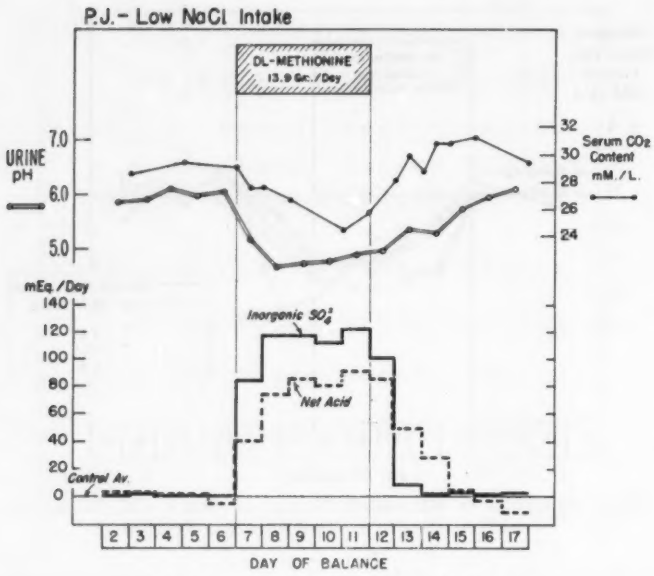

FIG. 2. SUBJECT P.J. LOW SODIUM INTAKE

Effect of methionine feeding on urine pH, serum CO_2 content and on the renal excretion of inorganic sulfate and "net acid."

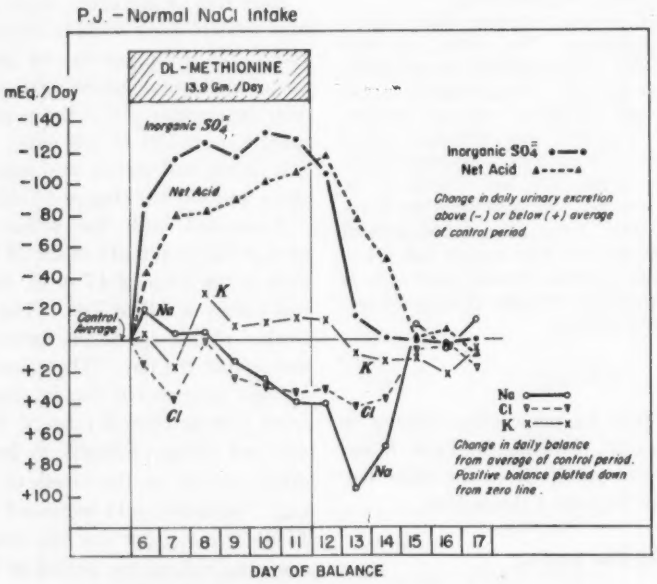

FIG. 3. SUBJECT P.J. NORMAL SODIUM INTAKE

Effect of methionine feeding on the daily balances of sodium, potassium and chloride and on the renal excretion of inorganic sulfate and "net acid."

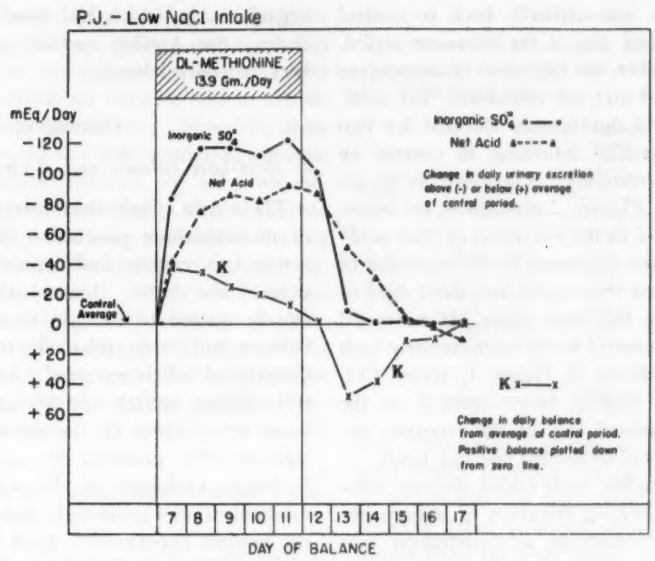

FIG. 4. SUBJECT P.J. LOW SODIUM INTAKE

Effect of methionine feeding on daily balances of potassium and on renal excretion of inorganic sulfate and "net acid." Sodium and chloride balances are omitted because renal excretion was always negligible.

acid" tended to lag behind the increase in inorganic sulfate.

There was no significant or consistent increase in excretion of sodium. In the two low-sodium balances urine sodium and chloride were negligibly small throughout the study. Of the other three balances with added salt, one (Table I) showed no significant change in sodium excretion, another a very small increase in sodium on the first day followed by subsequent retention (Figure 3), and the third a slight initial retention of sodium followed by apparently random fluctuations in balance. Cumulative sodium balances during the loading period, corrected for the control period, were + 67 mEq., + 50 mEq. and – 26 mEq., with corresponding chloride balances of + 84 mEq., + 136 mEq. and + 22 mEq.

Potassium excretion increased slightly in the balances with added salt, usually toward the end of the methionine period (Table I, Figure 3). Cumulative potassium balances, corrected for the control period, were – 55 mEq., – 49 mEq. and – 84 mEq. In the two studies on a salt-poor diet the potassium diuresis was prompt and of greater magnitude (Figure 4). Cumulative balances for the period, corrected for control, were – 111 mEq. and – 131 mEq.

Nitrogen excretion in the urine increased in each subject during the loading period by an amount greater than the increment in nitrogen intake represented by the methionine load. Thus, in every instance nitrogen balance became slightly negative during this period (– 8 to – 15 Gm., total).

Urinary calcium excretion was measured on several days in each metabolic period during three of the studies. It increased from control values of 1 to 3 mEq. per day to levels of 4 to 7 mEq. per day during the period of methionine loading (Table I). There were no marked or consistent changes in excretion of phosphorus.

Excretion of total organic acids showed a small but consistent increase in every study. The average increment above control ranged from 2 to 9 mEq. per day.

II. Recovery period

Immediately upon stopping methionine, the excretion of inorganic sulfate dropped sharply and

in every instance was virtually back to control levels by the second day of the recovery period. By contrast, however, the excretion of ammonium and titratable acid and the calculated "net acid" excretion remained significantly elevated for two or three days, usually returning to control by the fourth day of recovery. Therefore, as shown in Table I and in Figures 2 through 4, the increment above control in the excretion of "net acid" always exceeded the increment in the excretion of inorganic sulfate on the second and third days of recovery. During this time urine pH remained low, returning to control levels imultaneously with "net acid." As shown in Figure 1, serum CO_2 content was still slightly below control on the morning of the second day of the recovery period but was restored to normal one day later.

In the three studies with added sodium chloride there was a striking retention of sodium immediately after methionine administration was stopped, during the phase when the increment in the excretion of "net acid" exceeded that of inorganic sulfate (Table I, Figure 3). In each instance this positive balance of sodium was accompanied by a reduction in urine volume and a gain in weight of from 0.2 to 0.9 Kg. The cumulative sodium balances for the recovery period, corrected in each instance for the average daily balance during the control period, were: J.L., + 266 mEq.; P.J., + 180 mEq.; and L.S., + 210 mEq. At the end of the recovery period the final cumulative sodium balances for the entire study, including the control periods, were: J.L., + 102 mEq.; P.J., + 272 mEq.; and L.S., + 62 mEq. Chloride balances during recovery were also positive, but significantly less so than those of sodium. The corrected cumulative balances of chloride for the recovery period were: J.L., + 143 mEq.; P.J., + 130 mEq.; and L.S., + 115 mEq.

Potassium excretion dropped below control levels in every case within two or three days after stopping methionine, the retention being greatest in the two subjects on salt-free diets who had lost the most potassium during the loading period. Except for one subject (J.L., Table I) who tended to lose moderate amounts of potassium in his stools throughout the balance, potassium balances were essentially restored to normal by the end of each study.

The excretion of calcium, nitrogen and total organic acids, which had been slightly increased during the loading period, promptly dropped back to control levels.

DISCUSSION

1. Acid-base balance and electrolyte excretion

These data clearly demonstrate that large loads of DL-methionine produce a slight reduction in serum CO_2 content and a simultaneous acidification of the urine. Urine pH appeared to be closely related to changes in systemic acid-base balance and to be relatively independent of the quantity of sulfate excreted. Large loads of neutral sodium sulfate are known to produce intense acidification of the urine in salt-depleted subjects (9), probably by accelerating sodium-hydrogen exchange in the renal tubule. This mechanism was apparently not of importance in the present experiments, most likely because the sulfate load was relatively small.

Accompanying the acidification of the urine there was a slow progressive rise in the excretion of "net acid," chiefly in the form of ammonium. Although the increments in "net acid" always lagged behind the rise in sulfate, there was no demonstrable sodium diuresis. There was instead a diuresis of potassium (most marked with low-sodium diets) and calcium, and a slight reduction in chloride excretion relative to sodium. In this respect the effects of methionine would at first appear to be different from those of ammonium chloride, an initial sodium diuresis being a constant feature of the action of the latter compound in normal subjects on normal diets. However, no conclusions can be drawn, for there are no adequate data in the literature concerning the effects of ammonium chloride in doses less than 150 mEq. per day, whereas in the present experiments the maximum increments in sulfate were only 110 to 130 mEq. per day.

While the present experiments were being carried out, an independent study was conducted by Kaitz and Kass (10) for direct comparison of methionine with ammonium chloride. In each of two subjects they compared the effects of 150 mEq. ammonium chloride with those of 12 Gm. DL-methionine (which resulted in an average increment in urinary sulfate of 114 to 119 mEq. per day). There was little or no sodium diuresis with

either agent in those dosages. The initial effects of methionine on acid-base balance and renal acid excretion were, in general, similar to those they observed with ammonium chloride and to the effects of methionine reported here. However, urine pH tended to rise slightly with continued administration of ammonium chloride but not with methionine. They also observed a slight increase in phosphate excretion during methionine loading which was responsible for a relatively greater excretion of titratable acid. Their subjects ingested a considerably greater amount of phosphate than that which was fed in the present experiments, which may, in part, explain the negligible effect of methionine on phosphate excretion in these studies.

II. Relation between sulfate excretion and endogenous acid production

In the classical calculation of the acid equivalence of the diet ash it is implied that metabolism of dietary sulfur leads to the release of two equivalents of hydrogen ion per Mole of sulfur oxidized (1), and in theory such a result should be expected from the conversion of amino acid-sulfur to sulfate. Although it is well recognized that ingestion of sulfur-containing foods leads to increased acid excretion (2), there has never been any proof of the implied quantitative relationship between sulfur metabolism and the endogenous generation of acid. Since almost all of the oxidized sulfur is excreted as inorganic sulfate in the urine (11), and since all fixed acids are presumably excreted in the urine as "net acid," it should be possible in the present experiments to test the validity of the above concept by comparing the accumulated increments in "net acid" excretion with the increments in sulfate excretion after acid-base balance was restored to normal.

Table II compares these cumulative increments in sulfate and "net acid" in each of the studies. The final cumulative increments in "net acid" ranged from 79 per cent to 105 per cent of the cumulative increments in sulfate, with a mean value of 93 per cent. The close correspondence of these values would therefore suggest that the metabolism of methionine to sulfate does in fact yield two equivalents of hydrogen ion per Mole of sulfur so oxidized.

TABLE II

Comparison of cumulative increments in sulfate and "net acid"

Subject	Diet	Cumulative increment in urinary inorganic sulfate excretion	Cumulative increment in net acid excretion	Cumulative net acid as a percentage of cumulative inorganic sulfate
		mEq.	*mEq.*	%
J. L.	High NaCl	834	874	105
J. L.	Low NaCl	695	686	99
P. J.	High NaCl	833	747	90
P. J.	Low NaCl	666	524	79
L. S.	High NaCl	644	591	92
			Mean	93

A reasonable description of this quantitative relationship between the endogenous production of sulfate and the generation of acid can be constructed from a consideration of the reactions known to be involved in the metabolism of methionine (11). Figure 5 summarizes what now appears to be the major oxidative pathway for methionine and indicates the probable source of the acid. In the initial step, the details of which are not shown, methionine is demethylated and donates its sulfur to form cysteine. Neutral molecules are involved in this step. The remainder of the methionine molecule is oxidized to CO_2, urea and water, not shown in the figure. Cysteine is oxidized to cysteine sulfinic acid, ($pK = 2.1$), thus generating the first equivalent of hydrogen. Following deamination or transamination of cysteine sulfinate to β-sulfinyl pyruvate, a second equivalent of hydrogen is produced by the hydrolysis of the β-sulfinyl pyruvate to acid sulfite ($pK_2 = 5.2$) and pyruvate. The final oxidation of sulfite to sulfate does not yield additional acid. The potential alkali produced in the form of pyruvate is matched by the potential acid in the form of the amino group removed in the conversion of cysteine sulfinate to β-sulfinyl pyruvate. Thus, the net result of the degradation of 1 Mole of methionine or cysteine sulfur to sulfate is the release of two equivalents of hydrogen ion.

III. Sulfur balance

Approximately 70 per cent of the sulfur fed as methionine was excreted in the urine as inor-

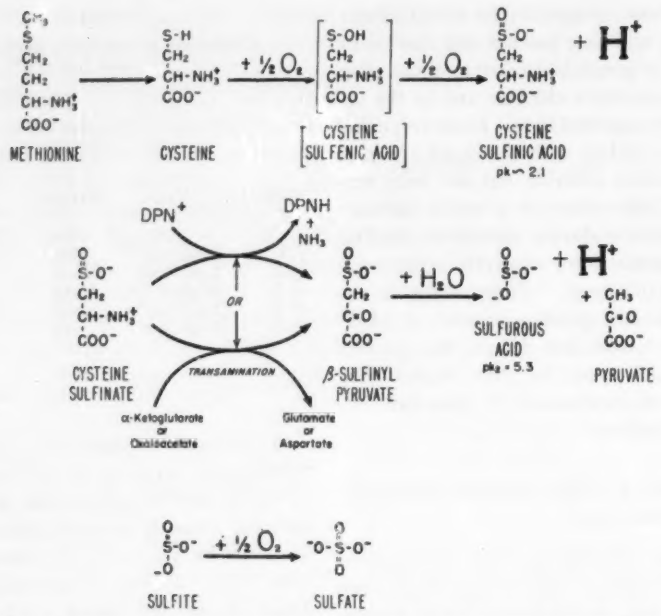

FIG. 5. SUMMARY OF MAJOR OXIDATIVE PATHWAY FOR AMINO ACID-SULFUR
See text for explanation.

ganic sulfate. There was no increase in urinary ethereal sulfur excretion during methionine loading (Table I) and no increase in the negligible quantities of inorganic sulfate excreted in the feces. Semiquantitative paper chromatographic studies in two balances indicated that an additional 10 to 15 per cent of the sulfur fed was excreted as unmetabolized methionine in the urine. No methionine was detected in chromatograms of the feces.

Thus, roughly 15 to 20 per cent of the administered load of methionine sulfur apparently followed some metabolic pathway other than that shown in Figure 5. Other possible metabolic fates of the sulfur-containing amino acids include: *a*) incorporation into tissue, *b*) deamination to analogous α-keto acids, *c*) oxidation to taurine and *d*) conversion to volatile compounds such as thiols or hydrogen sulfide. These possibilties are briefly considered below:

a) In view of the slightly negative nitrogen balances at the end of each study (− 1.0 to − 17.0 Gm.) it is unlikely that any significant quantity of sulfur was incorporated into tissue.

b) Total urinary α-keto acids were measured [2] in two balances and were negligibly small, although there was a slight rise during methionine loading. The maximum excretion never exceeded 1 mEq. per day. This suggests that formation of γ-methiol-α-keto butyric acid from methionine was not a major alternate pathway of methionine metabolism.

c) Further direct oxidation of cysteine sulfinate yields cysteic acid and finally taurine. No free taurine was found in the chromatograms of the urine in the two balances that were studied. Measurements of fecal taurine and taurocholate excretion were not made. However, in any event, taurine ($+ H_3N - CH_2 - CH_2 - SO_3^-$) is an internally neutralized substance and its formation should not affect acid-base balance.

d) Volatile sulfur-containing compounds such as methanethiol (CH_3SH) and methyldithiomethane (CH_3SSCH_3) have been found in the urine of patients with hepatic insufficiency (4, 12) who may have impairment of methionine degradation.

[2] We are indebted to Dr. W. E. Huckabee for these analyses.

Tests for these volatile substances were not made in the present experiments, but several observers noted that the subjects had the characteristic odor of hepatic coma while they were taking the methionine load, thus suggesting that volatile substances probably accounted for at least part of the missing sulfur. However, neither the thiols mentioned above, nor hydrogen sulfide, should have any influence on acid-base balance.

In summary, it would follow from these considerations that : *a*) most of the administered DL-methionine was metabolized, *b*) 80 to 85 per cent of the metabolized methionine went to inorganic sulfate, and *c*) the reactions leading to the formation of inorganic sulfate are the sole source of the endogenous acid derived from sulfur metabolism.

SUMMARY

Balance studies were carried out in normal subjects given 13.9 Gm. DL-methionine for periods of five to six days.

Administration of methionine produced small reductions in serum CO_2 content, associated with acidification of the urine and increased excretion of ammonium. There was a prompt sulfate diuresis, the total cumulative increment representing approximately 70 per cent of the administered sulfur, and 80 to 85 per cent of the methionine metabolized. During the loading period potassium excretion increased slightly, but there was no definite change in sodium balance. With recovery, both sodium and potassium were retained.

The changes in acid excretion were related to the observed reductions in serum CO_2 content and to the calculated acid retention. Excretion of acid, on the other hand, was relatively independent of the urine sulfate.

After final restoration of acid-base balance, accumulated increments in sulfate excretion were virtually equalled by the cumulative increments in excretion of "net acid." This strongly suggests the production of two equivalents of hydrogen ion for each Mole of oxidized sulfur, and confirms earlier views of the important contribution of sulfur metabolism to the endogenous fixed acid load.

Consideration of these data in the light of the known pathways of sulfur metabolism indicates the probable source of the hydrogen ions to be cysteine sulfinic acid and acid sulfite.

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PULMONARY TRANSCAPILLARY EXCHANGE OF Na^{24} AND P^{32}–LABELED PHOSPHATE IN PULMONARY EMPHYSEMA *

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In previous studies it was observed that 5 to 20 per cent of intravenously administered Na^{24} and P^{32}-labeled phosphate and other electrolytes escaped from the circulation during a single transit through the cardiopulmonary pool (1). The relatively small transcapillary exchange of electrolytes in the lungs as compared with skeletal muscle and certain other viscera was attributed to a lesser permeability of the pulmonary capillaries (1). The fraction of intravascular tracer electrolyte transferred across the pulmonary capillaries during pulmonary transit was diminished in the presence of a rapid circulation time and increased in patients with congestive heart failure and a prolonged circulation time (1). The present study extends these observations in patients with pulmonary emphysema where the problem of structural and physiological alterations of the pulmonary vascular tree is of particular interest.

METHODS

Subjects. The control subjects were adult patients, without evidence of pulmonary, cardiovascular or hematological disturbances, of the First Medical Service, Bellevue Hospital. Emphysematous patients without anemia or heart failure were selected from the Emphysema Section of the Chest Clinic, Bellevue Hospital. These patients had been observed for a period of years and their disease confirmed by pulmonary function tests and roentgenographic examination. The present study compares the exchange of Na^{24} ions in 11 control and seven emphysematous subjects and the exchange of P^{32}-labeled phosphate in six control and six emphysematous subjects.

The techniques employed in this laboratory have been described previously (1). Briefly, a solution containing either Na^{24} or P^{32}-labeled phosphate, together with a reference material (in these studies albumin-I^{131}), to which the capillaries are relatively impermeable, is injected rapidly into a peripheral vein and samples of arterial blood are obtained at two second intervals from an indwelling arterial needle previously inserted in the brachial artery of the opposite arm. The doses of the various isotopes used were as follows: Na^{24}, 40 to 80 μc.; P^{32}-labeled phosphate, 10 to 20 μc.; and albumin-I^{131}, 5 to 40 μc. The methods employed in assaying these isotopes have been described previously (1).

If a tracer electrolyte leaves the circulation during passage through the lungs the dilution curve shows concentrations which are lower than those for the reference substance on the ascending portion, owing to loss of electrolyte into the lungs during the initial transit. On the descending limbs of the curves the electroyte concentrations frequently approach or even exceed those of the reference material. This has been interpreted as being due to the return to the blood stream of some of the tracer electrolyte which has previously diffused out (1). At any time during the first circulation the net loss of the tracer electrolyte may be determined by comparing the areas under the curves up to that time. Since in many instances the descending portions of the curves reflect reentry of the electrolyte, it was felt that by comparing the areas under the ascending portions to the peak concentration (A%) a closer approximation of the magnitude of transfer would be obtained. However, both these ascending areas (A%) and the total extrapolated curve areas (T%) were used in the calculations (Table I).

All curves were plotted on semilogarithmic paper; the initial exponential portion of the descending limb was extrapolated to the baseline to exclude recirculation and the first circulation curve replotted on linear paper. Area measurements were made with a rotary planimeter. The difference in areas beneath the electrolyte and albumin curves was expressed as percentage of the albumin curve area. The mean circulation time was determined by the method of Hamilton, Moore, Kinsman and Spurling (2). Cardiac outputs were calculated by means of the arterial dilution technique (3, 4).

RESULTS

The mean net loss of Na^{24}, calculated from the ascending portion of the curves (A%) for seven emphysematous patients, was 3.8 ± 1.2 per cent (S.E.), while the mean loss for 11 control patients was 12.9 ± 1.6 per cent (S.E.) (Table I, Figures

* This material was presented in part at the Third World Congress of Cardiology, Brussels, Belgium, September 1958.

TABLE I

Pulmonary transcapillary exchange of Na^{24} and P^{32}-labeled phosphate

	Na^{24}			$P^{32}O_4$	
Subjects	A%*	T%*	Subjects	A%	T%
Control			Control		
Ni.†	21.3	6.0	Fi.†	6.0	12.0
Jo.†	19.4	12.0	Pa.†	20.0	19.3
Si.†	13.0	19.3	He.	10.9	9.0
Ki.†	6.0	5.0	Ju.	8.1	4.7
Hu.†	6.0	9.0	Ca.	12.1	8.4
Lo.	10.0	5.5	Re.	11.4	8.1
Co.	8.0	7.0			
Wa.	16.9	10.4			
Ro.	10.0	7.0			
Cas.	16.0	8.0			
Va.	15.5	12.3			
Mean values‡	12.9±1.6	9.2±1.5	Mean values	11.4±1.9	10.3±2.1
Emphysema			Emphysema		
Go.	0.4	6.0	Mo.	0.0	0.0
Ma.	1.3	3.3	Lu.	5.8	5.3
Su.	8.2	7.7	Mi.	5.4	3.9
Ba.	3.3	3.6	Ch.	4.1	3.9
Ron.	0.0	0.0	Tu.	0.0	0.0
Sh.	6.0	6.0	Mc.	0.0	0.0
Sc.	7.4	5.2			
Mean values	3.8±1.2	4.5±0.9	Mean values	2.6±1.2	2.2±1.0

* $A\% = \frac{I^{131}\text{ albumin ascending area} - \text{electrolyte ascending area}}{I^{131}\text{ albumin ascending area}} \times 100$;

$T\% = \frac{I^{131}\text{ albumin total area} - \text{electrolyte total area}}{I^{131}\text{ albumin total area}} \times 100.$

† Previously reported (1).

‡ Mean values expressed as mean ± standard error of the mean.

1, 2). The difference between the means is significant according to the "t" test (5) ($p < 0.01$).

Six studies were performed on emphysematous patients using P^{32}-labeled phosphate and albumin-I^{131}. Three of these subjects demonstrated no loss of phosphate during the entire first circulation, and the mean value for the group determined from the ascending curve areas (A%) was 2.6 ± 1.2 per cent (S.E.). The mean loss for P^{32}-labeled phosphate in six control subjects was 11.4 ± 1.9 per cent. These differences are also significant ($p < 0.01$).

In control subjects and in emphysematous patients the total Na^{24} curve areas (T%) were 9.2 ± 1.5 per cent (S.E.) and 4.5 ± 0.9 per cent (S.E.), respectively, less than the albumin curve area. The difference between the means is significant, the p value being between 0.05 and 0.01. In the control group the mean difference in areas beneath the total albumin-I^{131} and total P^{32} curves (T%) was 10.3 ± 2.1 per cent (S.E.), while in the emphysematous patients the mean difference was only 2.2 ± 1 per cent (S.E.). The difference between the means is statistically significant ($p < 0.01$).

The mean circulation time determined in 10 of the control subjects was 18.5 ± 0.9 seconds (S.E.) while that for all 13 emphysematous patients was 21.4 ± 1.2 seconds (S.E.) (Table II). The difference is of questionable significance, the p value being between 0.1 and 0.05.

The cardiac index for 12 of the control subjects in whom data were available was 3.99 ± 0.36 L. per minute per $M.^2$, while that for the 13 emphysematous patients was 3.69 ± 0.30 L. per minute per $M.^2$. The mean values do not differ significantly ($p > 0.1$). These values (particularly in Co., Mi. and Ron.) are somewhat higher than those reported by others (6, 7) for normal subjects. The explanation probably lies in the fact that no attempt was made to conform to basal resting conditions in either the control group or the emphysematous patients.

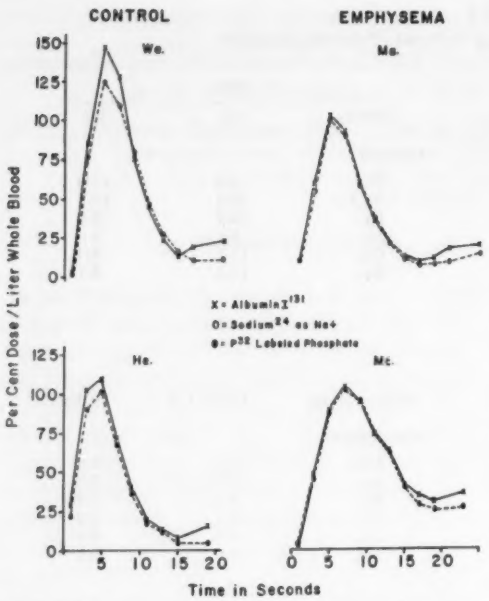

FIG. 1. SIMULTANEOUS ARTERIAL DILUTION CURVES

Albumin-I^{131} and Na^{24} as Na^+ in control and emphysematous patients (upper curves) and albumin-I^{131} and P^{32}-labeled phosphate in control and emphysematous patients (lower curves).

DISCUSSION

The results of the present study again indicate that the ion curves differ from the albumin curves in normal subjects. This difference partially or completely disappears in patients with pulmonary emphysema, indicating a decrease in the fraction of Na and phosphate transferred out of the blood during circulation through the lungs. Transfer of the tracer out of the circulation is primarily a function of capillary transit time, capillary surface area and capillary permeability. Although it is not possible to identify exactly alterations in any of these factors, it is interesting to speculate as to the mechanism involved in causing the diminished outflux in emphysema.

Although the capillary transit time was not determined in the present study, previous studies have demonstrated a correlation between circulation time and the fractional loss of plasma electrolytes (1). The calculated mean circulation time for the emphysematous patients was slightly longer than in the control subjects which would, if anything, tend to augment this fractional loss of plasma electrolytes (1). However, the capillary transit time has been calculated to be approximately one second (8) and alterations in the rate of blood flow through the capillaries could take place without being reflected in the calculated mean circulation time. On the other hand, it is unlikely that the lack of any detectable transfer could be accounted for solely by reduction in capillary transit time.

The effect of pulmonary emphysema on the capillary surface area is not known, but disruption and obliteration of the smaller blood vessels have been described (9–11) and a significant diminution in total capillary surface area would result in a decrease in outward movement of the tracer ions. Likewise, the diminished gas-diffusing capacity observed in emphysema has been partially attributed to a decreased membrane surface area (12–15). However, the finding of McNeil, Rankin and Forster (12) that the capillary blood volume remains essentially unaltered in emphysematous patients offers no support to the possibility that the capillary surface area is diminished but does not, of course, exclude it.

Capillary permeability remains to be considered. Alterations in the characteristics of the membrane have been discussed as another factor limiting gas exchange (12, 14) and it is possible that the decreased electrolyte transfer in emphysema could result from alterations in capillary permeability.

In addition to the three factors just discussed, other alterations in the emphysematous lung should be considered. The presence of right to left shunts bypassing the capillary bed could obviously limit outflux. However, such shunts are not characteristic of the emphysematous lung and, furthermore, would appear to be excluded by the absence of a rapid initial appearance of both tracer electrolytes and albumin-I^{131}. Bronchial arterial anastomoses with the pulmonary vessels have been described in chronic pulmonary disease (16–18), but since blood flow through these channels is from left to right no effect on the first circulation transfer would be expected.

Finally, it is conceivable that capillary permeability is so altered in pulmonary emphysema as to allow the tracer electrolytes and albumin-I^{131} to exchange at the same rate, thus eliminating any

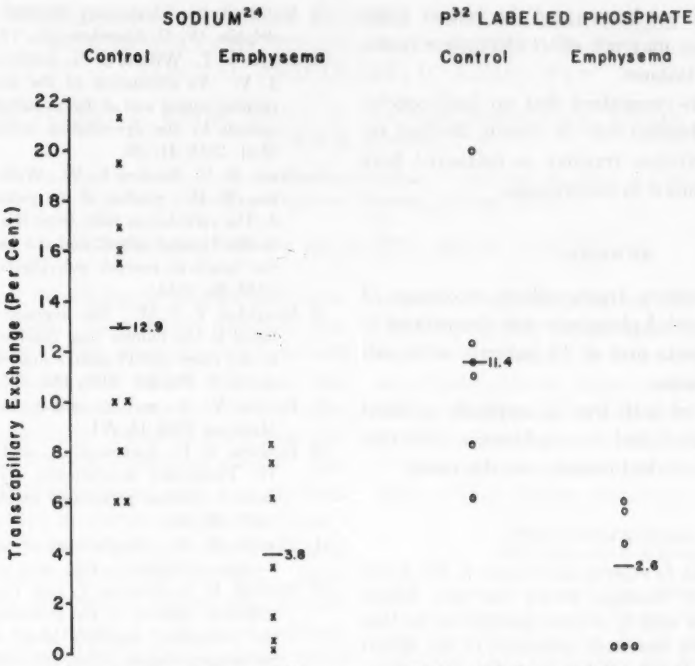

FIG. 2. TRANSCAPILLARY EXCHANGE OF Na^{24} AS Na^{+} AND P^{32}-LABELED PHOSPHATE

Exchange in control and emphysematous patients, expressed as per cent of plasma sodium and phosphate leaving the circulation in a single passage through the lungs.

difference in the curves. Although this possibility cannot be excluded, there is no evidence to support it.

The statements made in the discussion above are based on certain assumptions for which data are not available. Capillary transit time and capillary

TABLE II

Circulatory data

Control subjects	M.C.T.*	C.I.†	Emphysematous patients	M.C.T.	C.I.
	sec.	*L./min./M.²*		*sec.*	*L./min./M.²*
Ni.		3.30	Go.	19.4	4.17
Si.		3.84	Ma.	19.5	3.43
Lo.	17.9	4.67	Su.	17.6	3.63
Co.	14.0	6.70	Ba.	20.1	3.61
Wa.	17.3	3.40	Ron.	14.5	5.70
Ro.	24.2	3.40	Sh.	20.4	3.40
Cas.	17.5	3.92	Sc.	27.4	2.29
Va.	17.0	3.79	Mo.	18.0	3.34
He.	16.1	3.82	Lu.	28.2	2.49
Ju.	22.6	3.30	Mi.	24.7	6.00
Ca.	17.5	4.54	Ch.	26.4	2.84
Re.	21.0	3.21	Tu.	23.2	3.77
			Mc.	19.4	3.25
Mean values‡	18.5±0.9	3.99±0.36		21.4±1.2	3.69±0.30

* M.C.T. = mean circulation time.
† C.I. = cardiac index.
‡ Means expressed as mean ± standard error of the mean.

surface area are not known and the factors limiting gas exchange may not effect electrolyte transfer in a similar fashion.

Although it is recognized that no final conclusions as to mechanism may be drawn, the fact remains that electrolyte transfer as measured here is markedly reduced in emphysema.

SUMMARY

1. The pulmonary transcapillary exchange of Na^{24} and P^{32}-labeled phosphate was determined in 17 control subjects and in 13 patients with pulmonary emphysema.

2. Exchange of both ions is markedly reduced or practically abolished in emphysema. Factors causing this diminished transfer are discussed.

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METABOLIC PATTERNS DURING PHYSIOLOGIC SLEEP: I. BLOOD GLUCOSE REGULATION DURING SLEEP IN NORMAL AND DIABETIC SUBJECTS *

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Sleep is a time of varying physiologic and metabolic activity (2, 3). Many of the metabolic patterns which occur during sleep have not been well characterized. One area which falls into this category concerns the changes in blood glucose that occur during sleep. Previous studies on normal subjects have given conflicting results. Hyperglycemia, normoglycemia, and hypoglycemia have been reported to characterize sleep. Endres and Lucke (4) studied a group of adults and concluded that blood sugar rose during sleep. Similar results were reported by Chieffi and Rosselli del Turco (5) in a study of a group of children. Trimble and Maddock (6) studied capillary blood glucose concentrations in a group of nine normal young medical students. Their studies included eight isolated values taken during sleep and a series of values on a single subject taken at hourly intervals during an entire night's sleep. They concluded, although their data were inadequate for final conclusions, that blood glucose seemed to remain essentially constant during sleep. Dienst and Winter (7) reported that sleep was consistently associated with hypoglycemia and indeed that hypoglycemia was capable of inducing sleep. To our knowledge, no systematic study of blood glucose concentrations during sleep has been undertaken in a sizable group of subjects with diabetes mellitus.

Previous studies have had several limitations. Most workers have measured total blood-reducing substances rather than true glucose. The technique of blood sampling has not allowed continuing sleep by the experimental subjects. Conclusions have generally been based on single or isolated blood glucose values occurring during sleep. The present study was designed to obviate these limitations and had as its objective an investigation of blood glucose regulation in normal and diabetic subjects during physiologic sleep.

METHODS AND MATERIALS

All subjects reported to the laboratory approximately four to six hours following the evening meal. A brachial vein was intubated with a No. 14 polyvinyl catheter so that blood could be obtained for analysis without distracting or waking the patient. A comfortable bed in a dark environment was provided. Thirty minutes after the catheter was in position, a control blood was obtained for glucose analysis. The subject was then permitted to fall asleep and hourly bloods were obtained during the entire night; upon his waking in the morning, a final sample of blood was obtained. No sedative drugs were used. Between sampling, the catheter was kept open by means of dilute heparin. Blood glucose concentrations were determined in some patients by the glucose oxidase method of Froesch and Renold (8), and in others by the method of Nelson (9). The glucose concentrations reported are therefore "true glucose" and not total reducing substances. The following studies were performed.

In eight normal subjects, hourly blood sugars were determined during a given night of sleep.

A total of 16 diabetic subjects was studied by means of hourly blood glucose determinations during the night. Of these, five were untreated, except by diet; eight were insulin-treated and three were orinase-treated diabetics.

RESULTS

The data shown in Table I summarize the hourly blood glucose concentrations found at night in the normal subjects and show that hourly variations in blood glucose concentration are small during sleep. The greatest variation of blood glucose in

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† Public Health Service Research Fellow of the National Heart Institute.

TABLE I

Hourly blood glucose concentrations in eight normal subjects

Patient	P. M.		L. T.		R. A.		K. W.		G. M.		D. D.		J. C.		C. S.	
	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose
		mg. %		mg. %		mg. %		mg. %		mg. %		mg. %		mg. %		mg. %
11 p.m.			Awake	85											Awake	85
12 p.m.			Asleep	88	Awake	93			Awake	76			Awake	92	Awake	76
1 a.m.	Awake	78	Asleep	85	Asleep	91	Awake	95	Awake	73	Awake	81	Awake	96	Asleep	75
2	Asleep	72	Asleep	85	Asleep	89	Asleep	79	Awake	84	Asleep	93	Awake	88		
3	Asleep	72	Asleep	86	Asleep	84	Asleep	81	Awake	84	Asleep	88	Asleep	92	Asleep	76
4	Asleep	69	Asleep	83	Asleep	84	Asleep	84	Asleep	76	Asleep	67	Asleep	95		
5	Asleep	70	Asleep	83	Awake	80	Asleep	80	Asleep	77			Awake	89	Asleep	76
6	Awake	68	Asleep	83	Awake	89	Asleep	79	Asleep	78	Awake	75	Awake	95		
7	Awake	71	Asleep	83			Awake	74	Awake	71					Awake	68
8			Awake	79												

any normal subject was 26 mg. per cent. The mean standard deviation for the group as a whole was only ± 4.7 mg. per cent. Blood sugar concentrations during sleep are, therefore, characterized by their great constancy.

The hourly blood glucose concentrations on the diabetic subjects are summarized in Tables II, III and IV. These data indicate a much greater variability of blood glucose than is present in the normal subjects.

This variability may be most conveniently estimated by inspecting the data obtained on the untreated patients (Table II). In this group, the greatest variation of blood glucose concentration was 91 mg. per cent. The mean standard deviation of the group as a whole was ± 18.5 mg. per cent.

Many patterns of variation of blood glucose were found in the insulin-treated patients (Table III). Some of the more interesting patterns follow. Patient W.F. developed fairly severe hypoglycemia while asleep (blood glucose 32 mg. per cent). Despite hypoglycemia, he did not awaken. By morning, his blood glucose had returned to a normoglycemic level. Patient C.A. showed a progressive increase in blood glucose during the night, using from 155 to 293 mg. per cent during the night. Patient S.P. was hyperglycemic until 1:00 a.m. and from 1:00 a.m. until awaking in the morning he was normoglycemic. The nocturnal hyperglycemia was not reflected in the fasting morning blood glucose concentration. The greatest variation of blood glucose in any subject of this group was 140 mg. per cent and the mean standard deviation for the group as a whole was ± 27.5 mg. per cent.

The three orinase-treated patients showed a variability of blood glucose that was intermediate between the normal subjects and the untreated diabetics (Table IV). The mean standard deviation of hourly blood glucose concentrations in this group was ± 11.1 mg. per cent. Of interest are the data on Patient B.M. who was studied prior to and while on orinase therapy. Following orinase therapy, not only did the fasting blood glucose concentration fall but there was also a decrease in the variability of his hourly glucose concentration.

TABLE II

Hourly nocturnal blood glucose concentrations in five subjects with diabetes mellitus treated by diet alone

Time	J. W. Untreated		B. M. Untreated		R. C. Untreated		M. H. Untreated		A. B. Untreated	
	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose
		mg. %		*mg. %*		*mg. %*		*mg. %*		*mg. %*
11 p.m.	Awake	121	Awake	204	Awake	353	Awake	100	Awake	150
12 p.m.	Awake	125	Awake	182	Asleep	319	Awake	102	Awake	136
1 a.m.	Awake	121	Awake	164	Asleep	314	Awake	100	Awake	138
2	Asleep	185	Awake	158	Asleep	271	Awake	84	Awake	112
3	Asleep	120	Awake	175	Awake	276	Awake	98	Awake	122
4	Awake	124	Awake	150					Asleep	120
5	Awake	113	Awake	152					Awake	116
6			Awake	158					Awake	108
7					Awake	262	Awake	106		

DISCUSSION

The data presented in this paper establish that the hourly variation in blood glucose concentrations during sleep in normal subjects is extremely small. Blood sugar constancy during sleep must reflect an effective regulating mechanism since it is known that other factors capable of affecting glucose metabolism operate differently during sleep than during the waking state. For example, approximately 70 per cent of the daily adrenal secretion occurs between 12:00 midnight and 9 a.m. (10). Norepinephrine and epinephrine plasma levels are decreased during sleep (11). The constancy of blood glucose concentrations during sleep appears to be based on an effective regulating mechanism operating at a time when it does not have to deal with additional exogenous carbohydrates furnished by eating or increased glucose utilization produced by exercise.

In contrast, subjects with diabetes mellitus have blood glucose concentrations that are quite variable. This variability is present, first of all, in untreated diabetics. In some untreated patients, the variability may be explained by a decreased ability to utilize glucose. For example, Patient A.B. showed a progressive decline in hourly blood glucose concentrations from 11:00 p.m. to 6:00 a.m. the following morning. This decline may be related to impairment of glucose tolerance with the high glucose levels during the evening reflecting the hyperglycemic effects of the evening meal. On the other hand, Patients J.W. and M.H. showed spontaneous variations of blood glucose concentrations that could not be explained on this basis.

The greatest variability of blood glucose levels was found in the insulin-treated patients. A number of patterns of blood glucose variation was found which appear to be of clinical importance. The occurrence of hypoglycemia during sleep, without producing symptoms or arousal of the patient, is of obvious importance. This phenomenon has previously been reported by Maddock and Trimble (12). Patients who show significant hyperglycemia during a large part of the night, but who are normoglycemic by morning (Patient S.P.) would be considered well controlled by generally accepted criteria but, in truth, would be relatively uncontrolled for a significant portion of each 24 hours. Conversely, patients like C.A., whose glucose concentrations during sleep were considerably lower than during waking hours, might be overtreated on the basis of a morning blood sugar.

These data suggest that attempted diabetic regulation by means of urine glucose concentrations might be particularly unsatisfactory. Insulin dosages based on 24 hour glucose excretion might not correspond to the diurnal variations present in diabetics. Spot checks of urinary glucose might be even more unsatisfactory. For example, in Patient S.P. the morning urine would contain glucose that reflected nocturnal hyperglycemia. Such a patient would be given excessive insulin for his daytime needs.

It is important to emphasize that the diabetic patient is not only insulin-deficient but that he lacks an adequate mechanism for the regulation of blood glucose. In such patients, the physician attempts to substitute insulin in type and amount

TABLE III

Hourly nocturnal blood glucose concentrations in eight subjects with diabetes mellitus treated with insulin

Time	C. A. 30 U NPH 20 U CZI		B. S. 30 U NPH		W. F. 20 U NPH 20 U Cryst.		W. P. 20 U NPH		M. G. 20 U NPH		M. S. 46 U NPH		G. B. 25 U NPH		S. P. 40 U NPH	
	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose	State	Glucose
		mg. %		*mg. %*		*mg. %*		*mg. %*		*mg. %*		*mg. %*		*mg. %*		*mg. %*
8 p.m.													Awake	155	Awake	175
9													Asleep	155	Asleep	173
10													Asleep	148	Asleep	170
11							Awake	122	Awake	268			Asleep	125	Asleep	150
12 p.m.	Awake	155	Awake	124	Awake	83	Asleep	108	Awake	256	Awake	236	Asleep	135	Asleep	125
1 a.m.	Awake	168	Asleep	114	Asleep	61	Awake	70	Asleep	236	Awake	238	Asleep	102	Asleep	93
2	Asleep	162	Asleep	108	Asleep	42	Awake	88	Asleep	176	Awake	220	Asleep	102	Asleep	82
3	Asleep	175	Asleep	88	Asleep	34	Awake	92	Asleep	168	Awake	216	Asleep	93	Asleep	78
4	Asleep	194	Asleep	96	Asleep	38	Awake	90	Asleep	160	Awake	200	Asleep	105	Asleep	92
5	Asleep	230	Asleep	102	Awake	64	Awake	82	Awake	136	Asleep	204	Asleep	122		
6	Asleep	270			Awake	79	Asleep	90	Asleep	160	Asleep	186	Awake	123	Awake	103
7	Awake	293					Awake	82	Asleep	128	Awake	188				
8									Awake	140						

TABLE IV

Hourly nocturnal blood glucose concentrations in three subjects with diabetes mellitus treated with orinase

Time	B. M. 1.0 (Gm./day)		A. C. 0.5 (Gm./day)		M. H. 0.5 (Gm./day)	
	State	Glucose	State	Glucose	State	Glucose
		mg. %		*mg. %*		*mg. %*
11 p.m.			Awake	112	Awake	130
12 p.m.	Awake	124	Asleep	100	Asleep	124
1 a.m.	Awake	104			Asleep	124
2	Awake	94	Asleep	92	Asleep	106
3	Awake	96	Asleep	76	Asleep	106
4	Awake	100			Awake	110
5	Awake	94	Awake	72	Awake	106
6	Awake	96				
7	Awake	106			Awake	118
8					Awake	124

that will keep the patient's blood glucose at physiologic levels. However, the physician substitutes a biochemical substance and not a physiologic mechanism. Insulin will be released from the site of injection at a rate not determined by changes in blood glucose but at a rate determined by the type and amount of insulin. Since blood glucose concentrations of patients are not usually determined during sleep, the discrepancies between insulin dosage and insulin need might be expected to be great during this time. If one accepts the concept of ideal control of diabetes mellitus as meaning that the blood sugar level of diabetic subjects should correspond to the diurnal curves of normal individuals, then obviously greater attention must be paid to the hours of sleep in diabetics.

The data on the orinase-treated patients showed less variation than the data on insulin-treated patients. These data should not be interpreted as indicating that orinase is superior to insulin as a form of therapy. The orinase-treated patients had less severe disease than the insulin-treated patients. Nor does a relatively constant blood glucose level necessarily indicate more effective cellular utilization of glucose.

SUMMARY

1. Normal subjects show essentially constant blood glucose concentrations during sleep.

2. Patients with diabetes mellitus show marked variations in blood glucose concentrations during sleep.

3. Such variations have important therapeutic implications for the management of diabetic patients.

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A STUDY OF SUBJECTS WITH ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY. II. INVESTIGATION OF LEUKOCYTE ENZYMES *

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The susceptibility to hemolysis following administration of primaquine, naphthalene and other drugs, as well as after the ingestion of fava beans was described in individuals with a defect in their erythrocyte metabolism (1, 2). These erythrocytes manifest a number of abnormalities, namely, a low reduced glutathione (GSH) level, a glutathione instability when exposed to acetylphenyl hydrazine, a low glucose-6-phosphate dehydrogenase activity (G6PD), high aldolase and a decreased incorporation of glycine into GSH *in vitro* (3–7). The primary defect of these cells is probably related to the low G6PD.

This erythrocyte abnormality is transmitted by an incompletely dominant sex-linked gene (8, 9). Recently we demonstrated that the platelets of these individuals exhibit a decreased G6PD activity (10). On the other hand, Marks, Gross and Hurwitz have found a normal G6PD activity in the leukocytes of such individuals; the activity of this enzyme in their livers was found to be slightly decreased (11).

The investigation of G6PD in leukocytes in our population is described in the present communication. Contrary to the findings of Marks and associates, we found significantly lowered activity of this enzyme in leukocytes of subjects with the erythrocyte defect.

MATERIAL AND METHODS

Blood was obtained from: a) individuals, mostly males, whose erythrocytes demonstrated a marked glutathione instability by the method of Beutler, Robson and Buttenweiser (5) and very low G6PD activity (these subjects are referred to as "sensitive"); b) females with intermediate levels of this enzyme; c) control group.

Leukocytes were isolated from 20 ml. blood (12). The anticoagulant solution used was 1 per cent disodium sequestrene, 1 per cent tritone (W.R. 1339) in 0.7 per cent NaCl. All operations were performed at + 4° C. The blood was centrifuged for eight minutes at 200 G. The supernatant platelet-rich plasma was discarded. The buffy coat was removed, mixed with one-fifth of its volume polyvinyl pyrrolidone (Teva) and allowed to sediment for half an hour at + 4° C. in a tube at a 45° angle. The supernatant solution was removed and centrifuged at 250 G for 10 minutes.

In order to obtain a pure leukocyte suspension the remaining red cells were lysed by the addition of 3 ml. distilled water to the leukocyte sediment. After 20 seconds 1 ml. of 3.4 per cent NaCl solution was added in order to readjust the isotonicity of the medium. The mixture was centrifuged at 1,000 G for 10 minutes and the pure leukocyte sediment was resuspended in 0.5 ml. isotonic KCl, pH 7.4. The leukocytes were disrupted by freezing and thawing three times, using a mixture of dry ice and acetone. Leukocyte counts were performed before and after this procedure. No more than 5 per cent of cells remained intact. The leukocyte extract was spun down at 20,000 G for 10 minutes and the supernatant was used for enzyme determinations. Glucose-6-phosphate and 6-phosphogluconic dehydrogenases (6PGD) were determined by a slight modification of the methods of Kornberg and Horecker (13) and Horecker and Smyrniotis (14), respectively, as described in a previous communication (10). The volumes of the leukocyte extract used were such that the increase in the optical density at 340 mμ following reduction of triphosphopyridine nucleotide (TPN) was between 0.07 and 0.015 per 30 seconds. The results were expressed in units. A unit of enzyme activity is expressed as the change in optical density per minute per 10^8 cells (O.D. per minute per 10^8 cells) We used this type of calculation in order to compare our results with those of Marks and associates (11).

RESULTS

The results are summarized in Table I. The G6PD activity in leukocytes of the control group was found to be 34.7 ± 11.2 units, while in the "sensitive" group the respective mean was $7.59 \pm$

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2.72 units ($p < 0.001$), and in the intermediate group 13.29 ± 4.46 units. No significant difference in the 6PGD activity was detected between "sensitive" and nonsensitive subjects.

The G6PD and 6PGD activities are much higher in leukocytes than in platelets or erythrocytes. Table II illustrates the G6PD activity in leukocytes, platelets and red blood cells of normal and "sensitive" subjects.

Table III depicts the residual G6PD activity in leukocyte extracts heated for five minutes at 37° C. As can be seen, leukocyte extracts of "sensitive" subjects are more heat labile. However, since the initial enzyme levels in abnormal cells are much lower than those of the control group the validity of these results could be questioned.

TABLE I

Glucose-6-phosphate and 6-phosphogluconic dehydrogenase activities in leukocytes of normal and "sensitive" subjects

Subjects	Number	G6PD activity*	Number	6PGD activity*
Control	26	34.70±11.20	18	8.66±3.06
Severe deficiency red cell G6PD	25	7.59± 2.72	11	8.64±2.63
Intermediates	7	13.29± 4.46	2	9.00

* O.D. per minute per 10^9 leukocytes ± standard deviation.

DISCUSSION

Our increasing knowledge of the heterogeneity of enzymes raises two questions: a) Are the enzymes performing the same function in different tissues identical or different? b) Are they controlled by the same gene or do different genes regulate their functions?

In congenital galactosemia, the galactose 1-P uridyl transferase is absent in red cells and liver (15). In hypophosphatasia, the enzymatic defect was demonstrated in serum, liver, bone, kidney, intestine and white blood cells (16). We have demonstrated a decreased G6PD activity in platelets of subjects exhibiting this abnormality in their erythrocytes (10). Marks and colleagues, however, demonstrated a normal enzyme level in leukocytes of affected subjects and suggested that this enzymatic defect of the erythrocytes is not a primary but a more remote consequence of another genetic defect (11). On the other hand, we

TABLE II

Comparison of glucose-6-phosphate dehydrogenase activities in leukocytes, platelets and erythrocytes of normal and "sensitive" subjects

Subjects	Leukocytes*	Platelets*	Erythrocytes* †
Control	34.7 ±11.2	0.127±0.038	0.52±0.10
"Sensitives"	7.59± 2.72	0.024±0.017	0.08±0.02

* O.D. per minute per 10^9 cells ± standard deviation.
† Data obtained with a new batch of TPN on 16 controls and 5 "sensitive" males.

demonstrated a low enzymatic activity in leukocytes of our affected subjects. It is of great interest that the enzyme level in normal leukocytes is much higher than in erythrocytes or platelets. The enzyme activity of leukocytes and platelets of affected individuals is about 20 per cent of the normal activity. This could be related to the relatively similar survival time of platelets and leukocytes, which is of the order of 5 to 20 days, while the red cell survival is 120 days.

Since the 6PGD activity is the same in normal and affected cells, the differences in G6PD activity could not be related to an incomplete release of the enzyme into the extract. This conclusion is strengthened by the fact that in several experiments we calculated the enzyme activity on the basis of protein concentration in the extract and obtained the same results (17). To exclude the possibility that the difference was caused by an increased TPNH [1] oxidation in the extracts of affected cells, we measured TPNH oxidation rate of extracts obtained from platelets and leukocytes of normal and affected subjects and found practically no TPNH oxidation during a 20 minute

TABLE III

Residual glucose-6-phosphate dehydrogenase activity of leukocyte extracts heated at 37°C. for five minutes

Subjects	Number	Residual enzymatic activity in leukocytes
		%
Control	23	60–90
"Sensitives"	14	0–25

[1] We are indebted to Dr. Mager, Department of Biochemistry, Hebrew University Hadassah Medical School, Jerusalem, for his many valuable suggestions and for supplying us with TPNH.

incubation period. The experiment was performed under conditions identical to those for the G6PD assay (17).

We have no explanation at present for the discrepancy between our results and those of Marks and associates (11). It should be pointed out that we found previously a lower erythrocyte G6PD level in normal subjects and practically no enzyme activity in the erythrocytes of affected males. We are at present investigating this discrepancy. It appears that it was related to the TPN batch. We have lately investigated several batches of TPN and with one of the batches found erythrocyte enzyme values similar to those of Marks. These data are presented in Table II.

Our results show that the enzymatic defect in our population is not limited to the erythrocytes and can be demonstrated in platelets, leukocytes and probably in liver.

We investigated the G6PD and 6PGD activities on liver obtained during operation from two "sensitive" females and 10 controls and found values of 0.100 to 0.150 O.D. per minute per 100 mg. wet liver tissue in normals and 0.030 and 0.025 O.D. per minute per 100 mg. wet liver tissue in the "sensitive" subjects. The 6PGD activities were similar in both groups. It will be of interest to measure the enzyme level in other tissues. The obtained results would strengthen the hypothesis, previously suggested by us and others, that we are dealing with an enzyme that is qualitatively abnormal and becomes degraded more rapidly (10, 11, 18). This would explain our finding in "sensitive" subjects that a cell population with a low mean age, characteristic of platelets and leukocytes, has a higher enzyme activity than cells with a high mean age, for instance, erythrocytes. In order to demonstrate a qualitative difference of the enzyme we tried to investigate the heat stability of the leukocyte extracts. On incubating these extracts for five minutes at 37° C., we found a proportionally greater decrease of the enzyme activity in the extracts of abnormal cells than in normal cells. However, since the enzymatic activity in the affected cells is much lower than in normals, the interpretation of these results is difficult. Only isolation of the enzyme from normal and affected cells and its investigation will further our knowledge in this field.

SUMMARY

Glucose-6-phosphate (G6PD) and 6-phosphogluconic dehydrogenase (6PGD) activities were determined in leukocytes of individuals whose erythrocytes showed a deficiency of the former enzyme. Also a significantly lower level of G6PD was found in their leukocytes, whereas the 6PGD activity was found to be normal.

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CORTISOL METABOLISM IN MAN: OBSERVATIONS OF PATHWAYS, POOL SIZES OF METABOLITES AND RATES OF FORMATION OF METABOLITES *

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Variations have been shown to occur in the quantitative urinary excretion pattern of the metabolites of cortisol in a variety of traumatic events such as surgery, bone fracture and burns (1). The compounds evaluated were THF, allo-THF and THE,[1] and it was demonstrated that the relative proportion of THF: THE excreted during and after a "traumatic event" was markedly increased. In addition almost complete absence of allo-THF was observed in a chronically ill individual and it reappeared upon convalescence. The *rate of secretion of cortisol* was one factor involved in producing the increase in urinary THF: THE, since following the administration of adrenocorticotropin (ACTH) to normal individuals the relative proportion of urinary THF as compared with THE was observed to rise (1–5). However, gross changes in the pattern of urinary metabolites of cortisol occurred in "traumatized" individuals during periods of normal or even declining adrenal activity (1). This suggested that factors other than the rate of adrenal secretion were involved in altering the rates of excretion of the numerous metabolites of cortisol. It became clear that knowledge concerning the *pathways* of metabolism, the *pool sizes* of the metabolites and the relative *rates of formation* of the individual metabolites from cortisol would prove valuable in interpreting the urinary excretion patterns of these compounds.[2] Consequently these studies were undertaken to determine the extent of the reaction THF $\rightleftharpoons$ THE in volunteer subjects with and without the administration of ACTH; the pathways for the formation of THF, allo-THF, THE and cortolone from cortisol; the miscible pool size of THF, allo-THF, THE and cortolone; and the possibility of determining the overall rates of formation of THF, allo-THF and THE. This work was performed in volunteer subjects with cortisol-4-C^{14} as a tracer.

METHODS

General plan. Tracer doses of cortisol-4-C^{14} (1 μc., about 250 μg.) were administered intravenously to normal volunteer subjects and urine samples were collected every 15 minutes. The specific activity of cortisol and its metabolites was determined as a function of time, and pool sizes and pathways were determined by isotope dilution procedures after the intravenous administration of small amounts of unlabeled cortisol metabolites. The rates of formation of these metabolites were computed in several instances using the criteria described by Zilversmit, Entenman and Fishler (6).

Subjects. All subjects were normal healthy volunteers between 20 and 35 years of age. With one exception, all were male. Several of the subjects received an intravenous infusion of ACTH beginning four hours prior to the injection of radioactive cortisol and continuing throughout the experiment. The ACTH dose was calculated as 25 I.U. per eight hours. The remaining indi-

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[1] The compounds referred to are cortisol or F; cortisone or E; 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one, tetrahydrocortisol or THF; 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one, allotetrahydrocortisol or allo-THF; 3α,17α,21-trihydroxy-5β-pregnan-11,20-dione, tetrahydrocortisone or THE; 3α,11β,17α,20β,21-pentahydroxy-5β-pregnane or cortol; 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one or cortolone.

[2] The word *metabolism* as used denotes oxidation or reduction of the steroid molecule whereas *conjugation* is used to refer to the chemical binding of the steroid moiety with glucuronic acid.

viduals received no steroid or ACTH during the course of the experiment. Oral or intravenous fluids were used to maintain urine flow during the 15 minute collection periods.[3] For those not receiving ACTH the experiment was begun as soon as a satisfactory urine flow was obtained. All subjects were at bed rest for the duration of the experiment, except when they stood to void. An indwelling urethral catheter was employed in the female subject.

Steroids administered. In general, solutions of steroids for intravenous administration were prepared by weighing a known quantity of the compound into a clean vial, adding 1 to 2 ml. 70 per cent ethanol, capping the vial, and allowing it to stand overnight. The following morning 5 to 10 ml. sterile saline was added and the solution tested for sterility prior to use.

Cortisol-4-C^{14}. The cortisol-4-C^{14} was made available through the courtesy of the Endocrinology Study Section of the United States Public Health Service. One μc. of the compound corresponded to approximately 250 μg. of steroid and on chromatography, 92 to 94 per cent of the radioactivity migrated with cortisol while approximately 5 per cent of the radioactivity was more highly polar and about 2 to 3 per cent somewhat less polar than cortisol. Our analysis corresponds to that issued by the Endocrinology Study Section. The cortisol-4-C^{14} was used without further purification.

The THF and THE administered intravenously for the isotope dilution experiments were analyzed for several possible contaminants by chromatographing large quantities of the compounds and quantitatively analyzing specific contamination areas.[4] No significant amounts (less than 3 per cent) of THE or "20-hydroxylated compounds" were found in the THF and less than 1 per cent of THF or "20-hydroxylated compounds" was found in the THE. The β-cortolone was used directly.[5]

Analytic methods. Plasma and urines were extracted three times with double volumes of chloroform, the extract washed quickly twice with 1:20 volume 0.01 N sodium hydroxide and once with 1:20 volume of water. The solvent was dried with sodium sulfate and evaporated *in vacuo*. The extract containing the "free" (nonconjugated) F was chromatographed on paper overnight in the modified Bush (7) system, 3 volumes toluene: 1 volume 75 per cent methanol in water at 32°C. Quantitative fluorescence analysis for cortisol was carried out according to Sweat (8) and Peterson and Wyngaarden (9). Radioactive determinations were performed in a Nuclear-Chicago, D-47 windowless flow counter, 40 per cent efficient, at infinite thinness. Background was 20 counts per minute (cpm) and all samples were counted in duplicate with a maximal probable error of 3 per cent. Minimal counts collected for each sample were 1,280. All specific activities are expressed as cpm per microgram of steroid.

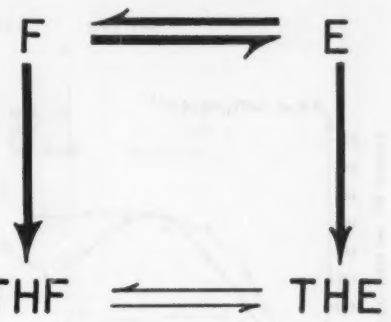


FIG. 1. SIMPLIFIED DIAGRAM OF ONE SEQUENCE IN METABOLISM OF CORTISOL IN THE HUMAN

To obtain the conjugated metabolites the urine samples were subsequently treated with β-glucuronidase,[6] at a final concentration of 200 Fishman units per ml. for three days at 37° C. at pH 5.0 or overnight at 47° C. and the same pH with 1,000 Fishman units per ml. The urine was extracted as described for the "free" fraction. The extract was chromatographed on paper for 39 hours in the toluene-methanol-water system. The THF, allo-THF and THE areas were located by dipping a 2 mm. wide center strip in alkaline blue tetrazolium. The peaks in blue formazan stain coincided with the location of radioactive peaks (1). The THF, allo-THF and THE areas were eluted with ethanol and their specific activities determined. The latter steroids were evaluated by the Porter-Silber reaction (10) using THF and THE as standards. The cortolone area[7] was located by eluting 1 cm. strips in the portion of the chromatogram corresponding to the migration of authentic β-cortolone. Aliquots of these eluates were counted and the radioactive peak was pooled, oxidized with periodic acid (11) and chromatographed for eight hours at 32° C. in a system composed of equal volumes of 67 per cent ligroin in toluene and 75 per cent methanol in water. The radioactive peak corresponding to the migration of 11-ketoetiocholanolone was eluted and its specific activity determined by using the Zimmermann reaction for the quantitative estimation

[3] At times large variations in the 15 minute urine output occurred even though fluid intake was regulated. However these variations did *not* alter the 15 minute excretion of metabolite. As an extreme example, in one individual 15 minute urine volumes showed a 10-fold variation from 12 ml. to 137 ml. while allo-THF excretion was 25 μg. and 27 μg. per 15 minutes, respectively.

[4] Quantitative determinations of THF and THE were carried out by the Porter-Silber reaction. The Zimmermann reaction, after periodic acid oxidation, was employed to evaluate "20-hydroxylated compounds."

[5] The β-cortolone was graciously supplied by David K. Fukushima and Thomas F. Gallagher of the Sloan-Kettering Institute for Cancer Research, New York.

[6] Ketodase®, a product of the Warner-Chilcott Laboratories, Warner-Lambert Pharmaceutical Co., New York, N. Y., was the β-glucuronidase preparation used.

[7] The α- and β-cortolone are not separated by this chromatographic system.

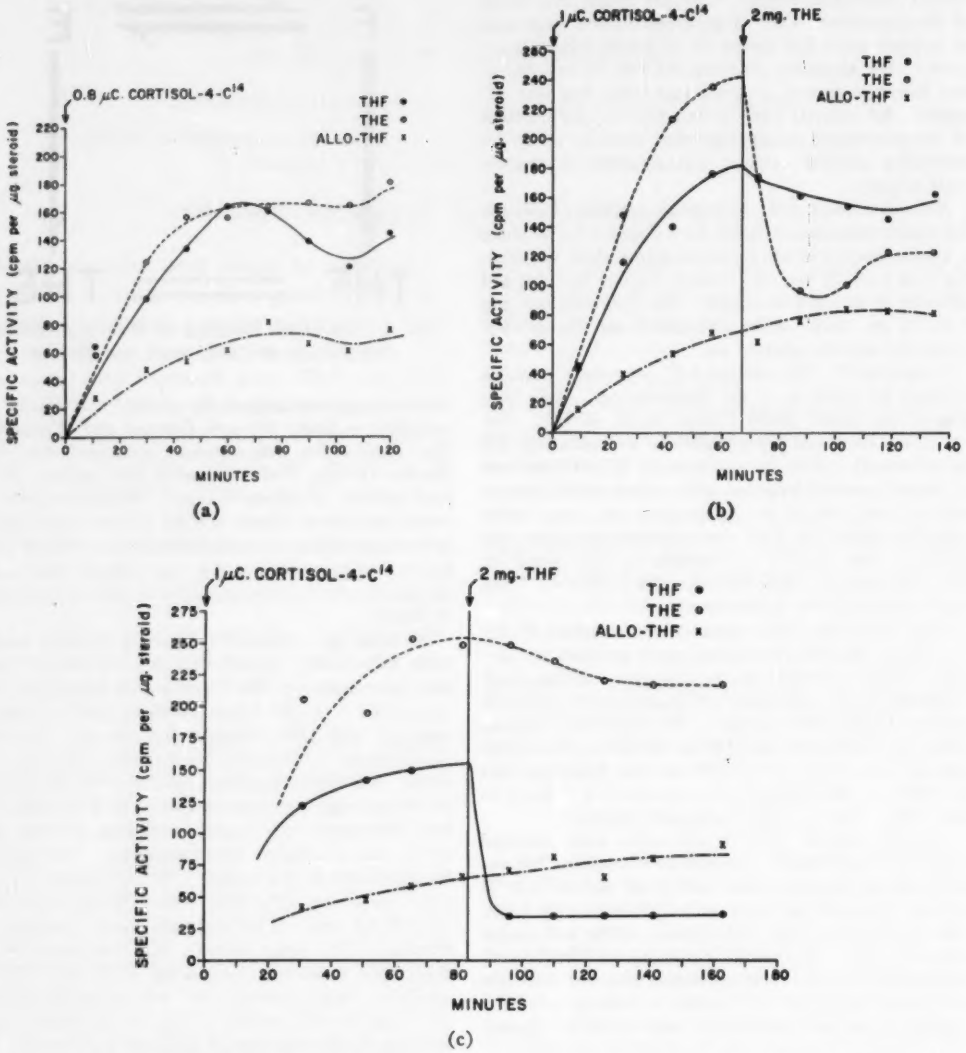


FIG. 2. SPECIFIC ACTIVITIES OF SEVERAL URINARY CORTISOL METABOLITES AFTER RAPID ADMINISTRATION OF TRACE QUANTITIES OF CORTISOL-4-C^{14} TO NORMAL MALE VOLUNTEERS RECEIVING NO EXOGENOUS ACTH

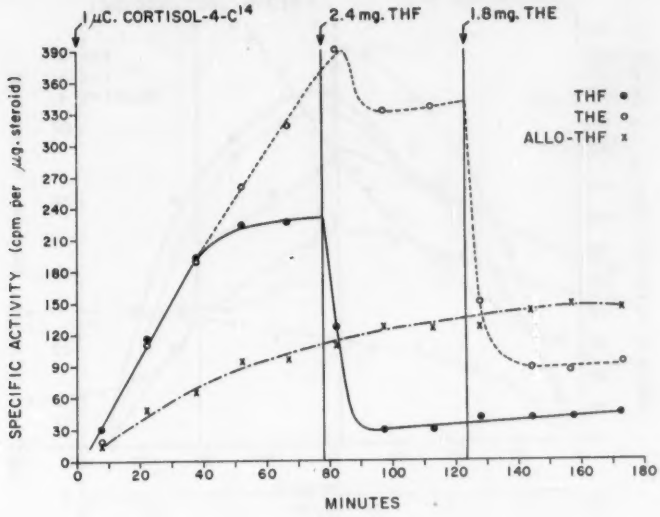
The data illustrate the small extent of the interconversion of the metabolites THF and THE.[1]

(a) D.B., age 22 years; no unlabeled metabolite given. The dip in specific activities at 100 minutes may be the result of a small increase in the rate of secretion of cortisol by the adrenal.

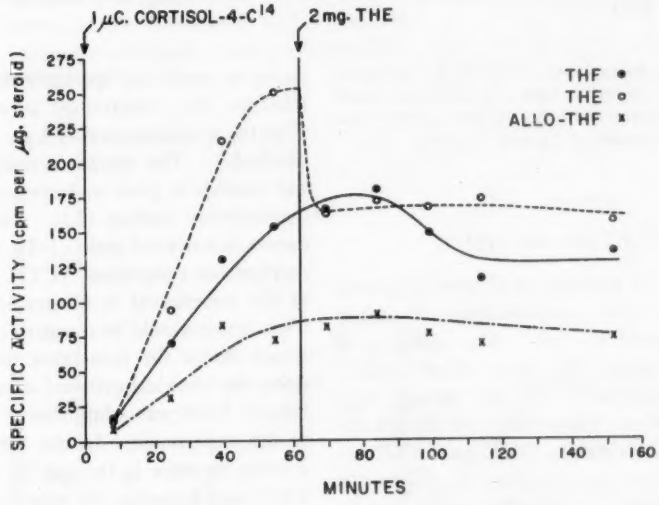
(b) D.B., age 22 years; unlabeled THE administered. Note the *minor* fall in specific activity of THF after the THE is administered. This suggests little conversion of THE to THF. Compare with (a).

(c) J.L., age 22 years; unlabeled THF administered. Note the negligible fall in specific activity of THE.

Figure 2 continued on following page



(d)



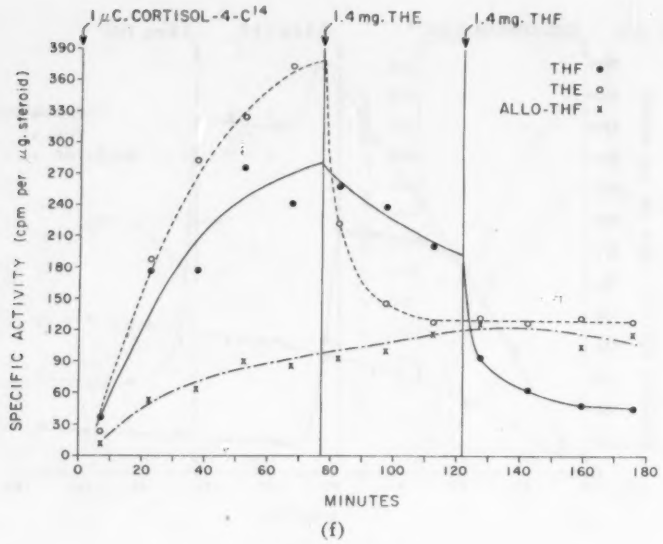
(e)

FIG. 2—*Continued*

(d) J.C., age 26 years; unlabeled THF and THE administered. Note some conversion of THF to THE.

(e) W.S., age 35 years; unlabeled THE administered. Note the relative lack of conversion of THE to THF.

Figure 2 continued on following page



(f)

FIG. 2—*Continued*

(f) E.K., age 21 years; unlabeled THE and THF administered. Note some conversion of THE to THF.

and expressing the micrograms of steroid as cortolone. Although authentic β-cortol separated distinctly from cortolone, no significant quantities of the cortols were found in the urine samples of Figures 5 and 8.

RESULTS

"Interconversion" of THF and THE

The reaction 11-hydroxy ⇌ 11-ketone occurs extensively after the administration of either cortisol or cortisone to man. The isolation of similar urinary metabolites after either cortisol or cortisone administration (12–14) strongly supports this contention. However, even though the chemical difference between THF and THE involves the same oxygen grouping at position 11 of the steroid nucleus as in compounds F and E, the two metabolites do *not* readily interconvert.

In the simplified diagram of Figure 1, which illustrates one major sequence in the metabolism of cortisol in the human, the formation of THF and THE are shown to proceed via F and E, respectively, while the interconversion of THF and THE is indicated as being minimal. Evidence that the interconversion of THF and THE is a minor pathway was obtained in several experiments in which the specific activities of these metabolites were determined as a function of time after the administration of 1 μc. cortisol-4-C^{14} (see Methods). The specific activity of each metabolite reaches a peak and then slowly declines in characteristic fashion (15). Such specific activity curves in a normal male (D.B., age 22 years) who received no exogenous ACTH during the course of the experiment is illustrated in Figure 2(a). This figure should be compared with Figure 2(b) which shows the data from the same individual using the identical protocol except that 2 mg. unlabeled THE was administered during the course of the experiment. In the latter figure there is a sharp decrease in the specific activity within the THE pool following the administration of the unlabeled THE, but there is little or no drop in the specific activity of THF as a consequence of the conversion of "cold" THE to THF. In the same type of experiment, Figure 2(c), unlabeled THF was administered intravenously to a normal male (J.L., age 22 years). This subject also did not receive exogenous ACTH. The decline in specific activity of the THF pool is not accompanied by any observable decrease in the THE specific

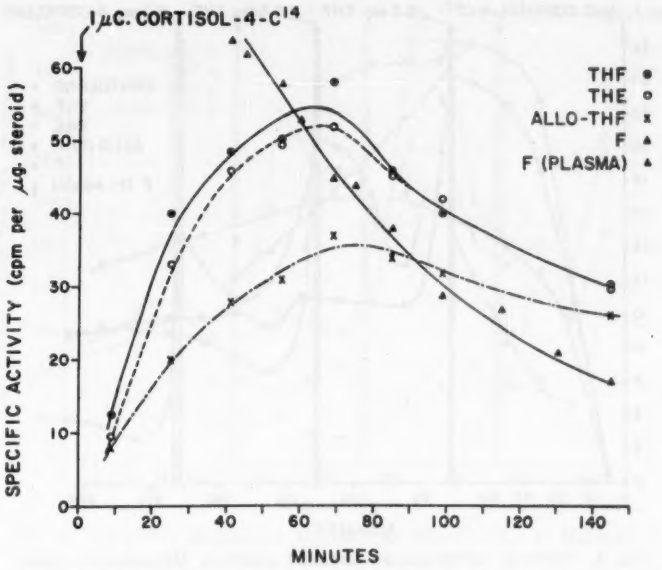


FIG. 3. SPECIFIC ACTIVITIES OF URINARY CORTISOL METABOLITES AFTER RAPID ADMINISTRATION OF 4-C^{14}-CORTISOL TO A NORMAL MALE, J.C., AGE 26 YEARS, DURING CONTINUOUS ACTH INFUSION

Compare with Figures 5 and 8 for conversion of metabolites during ACTH administration.

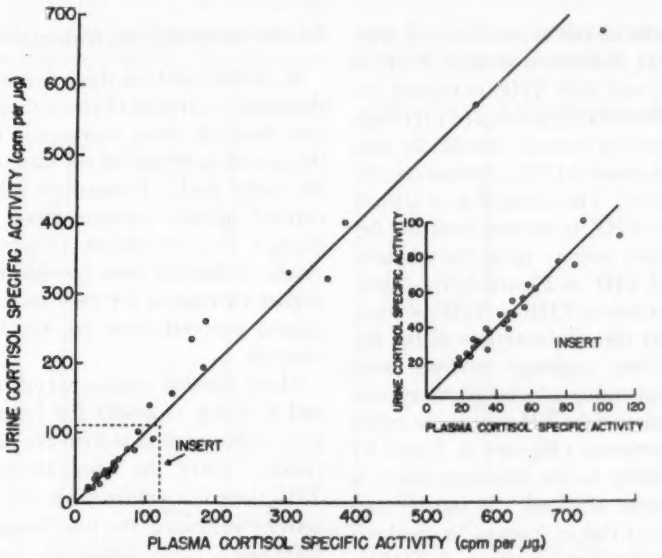

URINE CORTISOL SPECIFIC ACTIVITY (cpm per μg)
PLASMA CORTISOL SPECIFIC ACTIVITY (cpm per μg)
INSERT
URINE CORTISOL SPECIFIC ACTIVITY
PLASMA CORTISOL SPECIFIC ACTIVITY

FIG. 4. RELATIONSHIP BETWEEN URINE AND PLASMA CORTISOL SPECIFIC ACTIVITIES

Data taken from six normal volunteer subjects. The straight line was drawn with a slope of 1.

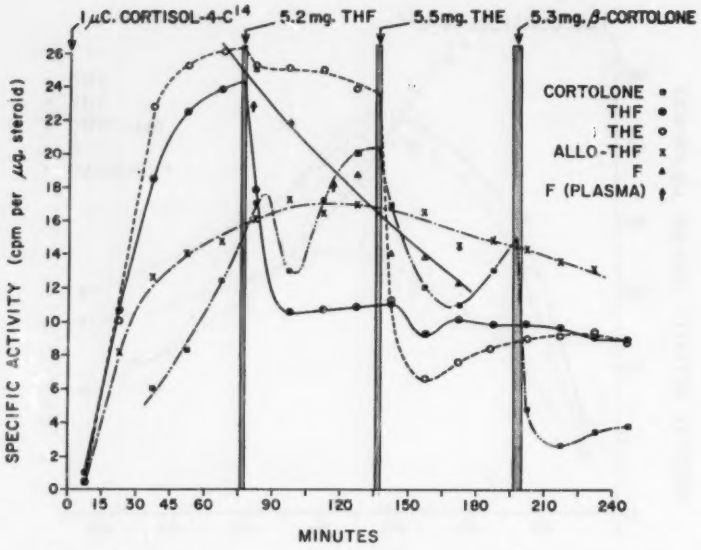


FIG. 5. SPECIFIC ACTIVITIES OF URINARY CORTISOL METABOLITES AFTER RAPID ADMINISTRATION OF 4-C^{14}-CORTISOL TO A NORMAL FEMALE, M.L.P., AGE 24 YEARS

Subject received continuous ACTH infusion throughout the experiment. The data, which indicate the relative conversions of the metabolites, were used to compute pool sizes and rates of formation of metabolites. Refer to Figures 6 and 7 for additional data from this experiment.

activity. The results of the experiments of Figures 2(b) and 2(c) indicate that little THF is formed from THE and little THE is formed via THF. The experiments of Figures 2(d) through 2(f), also performed in normal individuals who did not receive exogenous ACTH, confirm the observations cited above. The conversion of a small amount of THF to THE is evident from the decline in THE specific activity upon the administration of unlabeled THF in Figure 2(d). Similarly a small conversion of THE to THF is noted in Figure 2(f) after the administration of the unlabeled THE. When volunteer subjects were maintained at a high steady adrenal secretory rate by continuous infusion of ACTH during the entire course of the experiment (Figures 3, 5 and 8) similar results relating to the interconversion of THF and THE were obtained. It can be concluded (see Figure 1) that in "normal" individuals the major pathway for the formation of THF is via F and the major pathway for the formation of THE is via E. Only minor interconversions of THF and THE occur (16, 17).

Further metabolite interconversions

In studies such as these one must assume that the specific activities of compounds obtained at any time from the urine represent a true sampling of the specific activities of the same compounds from the body pool. Fortunately, plasma and urine cortisol specific activities from six subjects do show a 1:1 correlation (Figure 4). Unfortunately, it has not been possible to demonstrate a similar correlation for each *metabolite* since their plasma concentrations are too low for accurate analysis.

More detailed studies are shown in Figures 5 and 8, which represent the "steady state" condition in two normal individuals during ACTH infusion. After the administration of unlabeled THF there is a sudden fall in the cortolone specific activity indicating the transformation of THF to cortolone. The subsequent rise in cortolone specific activity prior to the administration of unlabeled THE is due to the continued transformation of cortisol to cortolone via intermediates

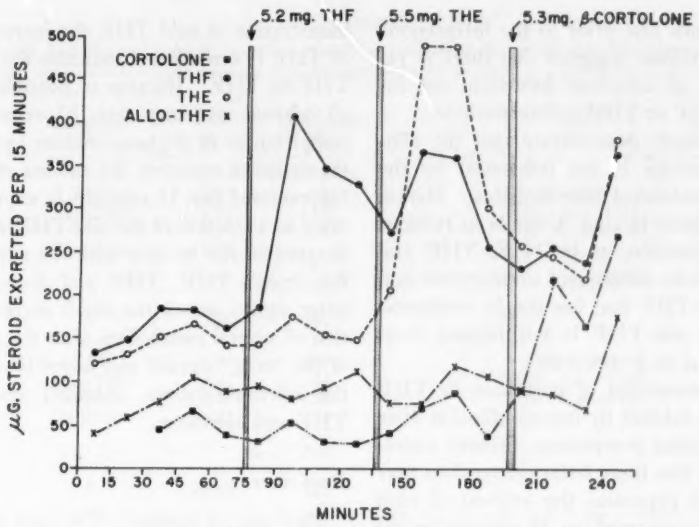


FIG. 6. QUANTITY OF URINARY METABOLITES EXCRETED PER 15 MINUTES IN EXPERIMENT OF FIGURE 5

other than THF. Following the administration of unlabeled THE there is another decrease in cortolone specific activity which is consistent with the observation (18, 19) that THE is also a precursor of cortolone. Quantitative values for the *extent* of conversion of THF and THE to cortolone are not deducible from Figures 5 and 8. The decreases in cortolone specific activity occur abruptly and rise sharply. There is no indication that simple isotope dilution calculations can be applied under these circumstances. Consequently the data do not make it clear whether THF proceeds to cortolone via THE or perhaps an unknown pathway. The rather sharp increase in cortolone specific ac-

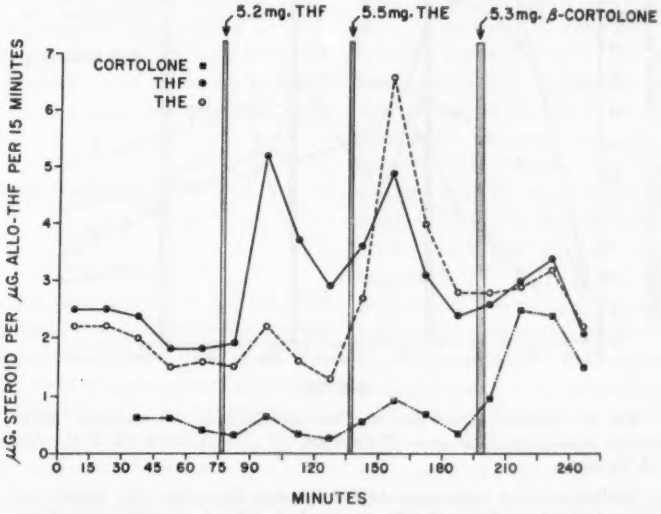


FIG. 7. QUANTITY OF URINARY METABOLITES EXCRETED PER 15 MINUTES IN EXPERIMENT OF FIGURE 5, EXPRESSED AS A FUNCTION OF ALLO-THF[1]

tivity, which occurs just prior to the intravenous addition of β-cortolone, suggests that there is yet another pathway of cortolone formation not involving either THF or THE as intermediate.

The figures clearly demonstrate that the allo-THF specific activity is not influenced by the addition of the unlabeled intermediates. Hence, once the double-bond in ring A has been reduced to the 5β configuration, as in THF, THE and cortolone, there is no subsequent unsaturation and formation of allo-THF that has the 5α configuration. Therefore allo-THF is not formed from either THF, THE or β-cortolone.

Although a conversion of cortolone to THF and THE is not evident by isotope dilution after addition of unlabeled β-cortolone, urinary excretion data suggest that these latter conversions may occur. Figure 6 expresses the *amount* of each urinary metabolite excreted per 15 minutes for the subject of Figure 5. Small variations in the 15 minute excretions of the metabolites prevent a clear visualization of the conversion of one metabolite to another. As an exception, after the administration of cold THE the increased excretion of THF is evident and indicates the conversion of THE to THF. Because of possible variations in *a*) adrenal secretory rate, *b*) renal function, *c*) completeness of β-glucuronidase hydrolysis or *d*) the extent of recovery, the amount of each metabolite excreted per 15 minutes is expressed in Figure 7 as a fraction of the allo-THF whose quantity is assumed not to vary with the administration of the "cold" THF, THE and β-cortolone. This latter figure shows the small increases in excretion of several metabolites after the administration of the "cold" steroid and hence is consistent with the interconversions, although small, of THF, THE and cortolone.

Pool sizes

Pool size of cortisol. The pool size of cortisol was calculated by dividing the total radioactivity injected (corrected to 92 per cent purity) by the specific activity of the cortisol extrapolated to time zero. The latter value was obtained from a

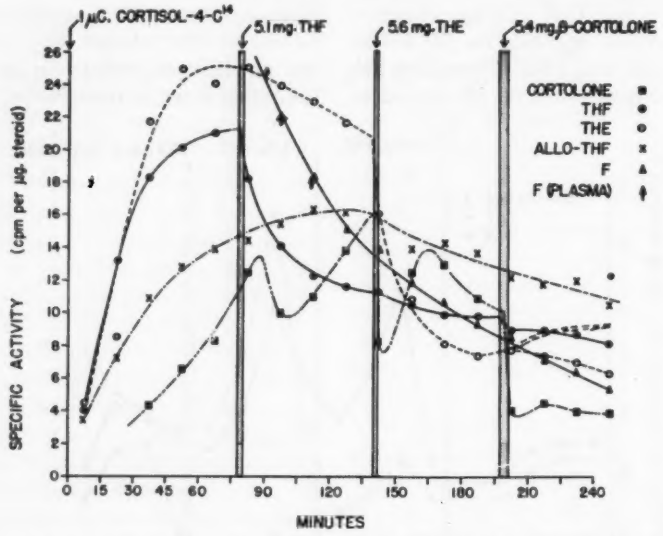


FIG. 8. SPECIFIC ACTIVITIES OF URINARY CORTISOL METABOLITES AFTER RAPID ADMINISTRATION OF 4-C^{14}-CORTISOL TO A NORMAL MALE, E.K., AGE 21 YEARS

Subject received continuous ACTH infusion throughout the experiment. Note similarity to Figure 5. The double dashed line is a crude extrapolation of the THE curve as it might appear if the added β-cortolone were not converted to THE (refer to Results, *Pool Sizes*).

TABLE I

*Readily miscible pool size and rates of formation of cortisol metabolites during adrenocorticotropin administration**

Patient (a)	Compound (b)	Pool size† (c)	Rate of formation* (d)	Metabolite ratio (e)	Ratio from calculated pools (f)	Ratio from urinary excretion (g)
		mg.	*mg. per 24 hr.*			
M. L. P.	THF	3.6	65	THF : THE	1.1	1.1
female	Allo-THF	2.1‡	18	THF : Allo-THF	1.7	1.9
24 years	THE	3.4	72	THF : Cortolone	3.3	3.7
	Cortolone	1.1§				
			Sum 155			
	F	17.5‖	195			
E. K.	THF	4.8	50	THF : THE	1.2	1.1
male	Allo-THF	4.4‡	33	THF : Allo-THF	1.1	1.1
21 years	THE	4.0	81	THF : Cortolone	4.0	4.7
	Cortolone	1.2‡§				
			Sum 164			
	F	16.6‖	211			

* Refer to Methods and Results for details. "Steady state" plasma cortisol concentrations, in micrograms per 100 ml. plasma, were 51 for M. L. P. and 37 for E. K.

† Calculated by isotope dilution unless otherwise indicated.

‡ It can be seen in Columns (f) and (g) that the relative amounts of the metabolites, THF and THE, excreted per 15 minutes as the glucuronides, are proportional to the size of the pools calculated by isotope dilution. Hence, "a" = kN, where "a" is the amount of metabolite excreted per 15 minutes, N is the pool size and k is a proportionality factor; k is 3.6×10^{-3} per 15 minutes for M. L. P. and 2.2×10^{-3} per 15 minutes for E. K. Knowing k and "a," pool sizes were calculated for allo-THF and cortolone.

§ Cortolone includes both 20α and 20β isomers.

‖ Total radioactivity injected divided by cortisol specific activity extrapolated to time zero. Cortisol half-life for M. L. P. is 88 minutes, for E. K., 78 minutes.

method of least squares plot of the log (cortisol specific activity) as a function of time.

Metabolite pool size, by isotope dilution. The fall in specific activity after the addition of nonlabeled metabolite can be used to determine the readily miscible pool size of a given cortisol metabolite by applying the following general isotope dilution formula:

$$N\ (s.a.)_0 = (N + n)(s.a.)_1$$

or

$$N = \frac{n\ (s.a.)_1}{(s.a.)_0 - (s.a.)_1}$$

where N = miscible pool size in milligrams prior to addition of diluent; n = milligrams unlabeled diluent added to pool (corrected for a small loss of diluent excreted and for conversion of unlabeled diluent to other metabolites); $(s.a.)_0$ = specific activity prior to addition of diluent; $(s.a.)_1$ = specific activity after addition of diluent.

The value for the specific activity of the metabolite *after mixing with the unlabeled diluent,* $(s.a.)_1$, is taken at the region in which the specific activity reaches a plateau. It is assumed that at this plateau the added metabolite is homogeneously mixed with all or the major portion of the metabolite pool. The latter is sometimes referred to as the "readily miscible pool." In some instances the plateau is clearly defined; in others it is not. As an example, in Figure 5, the THF plateau is apparent 30 minutes after the administration of "cold" THF, whereas the THE plateau is not reached until 75 minutes after the addition of unlabeled THE. Unfortunately, in the THE curve of Figure 8 the "plateau" after the administration of "cold" THE is not reached prior to the injection of β-cortolone. Our observations on the conversion of β-cortolone to THE would explain the failure of the THE curve to attain the expected plateau. Consequently, it was necessary to extrapolate roughly the expected THE "plateau." This is indicated by the double lines during the last period of Figure 8. In the future, increasing the time interval between additions of cold diluent will assist greatly in locating these plateaus without resorting to extrapolations.

Metabolite pool size calculated from urinary excretion data. It is difficult to determine pool size by isotope dilution procedures in those instances when metabolites are not available as diluents or when it is not possible to locate the plateau after dilution. In such situations it is possible to estimate pool size if the rate of excretion of urinary metabolites is known. In the "steady state" the rate of excretion of metabolites is relatively constant prior to the administration of diluents (Figure 6). If the metabolite pool exists mainly in the form of metabolite-glucuronidate, then the rate of excretion of each metabolite-glucuronidate should be proportional to the pool size. Hence, $a = kN$, where "a" is the amount of metabolite excreted per 15 minutes, k is a proportionality factor and N is the pool size. In each of the two experiments in Table I, k was found to be identical for THF and THE. For subject M.L.P., k was 3.6×10^{-2} per 15 minutes; for E.K., k was 2.2×10^{-2} per 15 minutes. That the same values of k were obtained for THF and THE merely implies that once conjugation with glucuronic acid has occurred the steroid-conjugate is so highly polar that small differences in the steroid moiety play little or no role in determining the rate of excretion of the steroid-conjugate. This implies that, in any given individual, k is identical for all cortisol-metabolite-glucuronidates.

By substituting the measured value of "a" and the value of k, previously calculated for THF and THE, N (the pool size) was determined for allo-THF in both subjects of Table I and for cortolone in Subject E.K. It can be seen, Column (g), that the relative proportions of the metabolites excreted per unit time, *are* equal to the ratio of the calculated pool sizes of the same compounds, Column (f). This is consistent with the assumptions made concerning the relationship of pool size to the rate of excretion of steroid-glucuronidate.

Rates of formation of metabolites. A method for computing the rate of formaton of a metabolite from its precursor in a biologic system has been clearly described by Zilversmit and associates (6). It is necessary to assume that the metabolite is in the steady state during the interval of the study, that is, its rate of appearance must equal its rate of disappearance and this rate must be constant. In addition, it is imperative that random treatment of all molecules occur. If these assumptions hold, then, in a graph of the specific activities of precursor (cortisol) and metabolite as functions of time, the specific activity curve of the precursor will intersect the specific activity curve of the metabolite at the maximum of the metabolite curve. The data of Figure 3, in which no unlabeled metabolites were administered, as well as the data of Figures 5 and 8, meet these criteria for THF, allo-THF and THE. Consequently Zilversmit's formulas were applied to calculate the rates of formation of THF, allo-THF and THE. The formulas are as follows:

$$p = \frac{N}{t_t} \quad \text{and} \quad t_t = \frac{A}{m}$$

where p = rate of formation of metabolite (amount formed per unit time); N = pool size of metabolite (amount); t_t = turnover time (time necessary to form an amount of metabolite equal to the pool size); A = area (determined graphically) *between* the specific activity curves (as a function of time) of the precursor and metabolite during an arbitrary time interval, $t_2 - t_1$. The units of A are specific activity $\times$ time; m = difference between the specific activities of the metabolite at t_2 and t_1.

The turnover, or rate of secretion, of cortisol, which in the steady state is equivalent to the amount of cortisol removed from the body pool per unit time either by conversion to metabolites or by excretion as "free" cortisol, was calculated as $\ln 2/t_{1/2} \times$ pool size where $t_{1/2}$ is the biological half-life of the isotopically-labeled cortisol (20). The biological half-life, or the time necessary to reduce the specific activity of the cortisol pool to one-half value, was obtained from the slope of the straight line, computed by the method of least squares, of a plot of the log of the specific activity of cortisol as a function of time.

The rates of formation, p, for the various metabolites are shown in Table I, Column (d), along with the rate of secretion of cortisol. It can be seen that the sum of the rates of formation of the three metabolites, THF, allo-THF and THE, represent approximately 80 per cent of the total turnover of the secreted cortisol. These metabolites, in turn, may be converted in part to other compounds. Data herein presented suggest that THF and THE may be transformed into cortolone. It seems logical that the former two metabolites may

also be converted to ketosteroids (21) and to the cortols (22). Since the size of the metabolite pool that exists during the steady state is the resultant of the rate of formation of this metabolite minus its rate of excretion plus transformation to other steroids, there may be considerable difference between the relative pool sizes of the metabolites and the relative rates of formation of the same compounds. For example, in Table I, it is clear that even though the ratio of the sizes of the THF and THE pools is approximately 1:1 for both subjects, the *rates of formation* of THF are significantly *lower* than the rates of formation of THE and hence the smaller pool size of THE is indicative of the transformation of this metabolite to other steroids.

The sum of the rates of formation of the metabolites THF, allo-THF and THE total about 50 mg. less than the turnover of cortisol. Hence certain of the metabolites of cortisol may not pass through the aforementioned three metabolites as intermediates. It may be inferred that this difference of approximately 50 mg. will include small amounts of cortisol itself, cortisone, a portion of the cortols and cortolones, 20α-hydroxy-cortisol (23, 24), 20β-hydroxycortisol (24, 25), 6β-hydroxycortisol (26), 11 ketoandrosterone (21) and a portion of 11β-hydroxyandrosterone (21).

DISCUSSION

Evidence presented in this study indicates that two of the major metabolites of cortisol, THF and THE, are derived essentially from cortisol and cortisone, respectively. Although there is some interconversion of THF and THE, this does not appear to be a major pathway for the formation of either THF or THE. It cannot be implied that enzyme systems capable of transforming THF $\rightleftharpoons$ THE are actually *deficient*. If conjugation with glucuronic acid prevents further transformation of the steroid then it is possible that the *rate of conjugation* of the cortisol metabolites may be an important factor in limiting the interconversion of certain of the metabolites. In addition, there is no perceptible conversion of THF, THE or β-cortolone to allo-THF, suggesting that once the double bond of ring A of the steroid nucleus has been reduced to form compounds such as THF and THE with the 5β configuration, there is no unsaturation with subsequent reduction of the double bond to the 5α or "allo-" configuration. Further evidence suggests the *in vivo* interconversion, to a small extent, of the compounds THF $\rightleftharpoons$ cortolone and THE $\rightleftharpoons$ cortolone.

The kinetic methods employed in this study involve isotope dilution procedures carried out within a complex body pool. The kinetics of the distribution of chemical substances within biological compartments have been described by numerous investigators (15, 20, 27). Certain aspects of the kinetics of distribution of cortisol in man have been discussed by Hellman, Bradlow, Frazell and Gallagher (28) and Peterson and associates (29), while other studies (30–32) have been concerned with measurements of the "half-life" of plasma cortisol in various physiological conditions. Implicit in these studies is the concept that if first-order kinetics prevail then half-lives can be computed. Unfortunately such studies do not yield any information concerning pool sizes of various steroids, rates of formation of individual metabolites or the various chemical pathways of metabolism that occur. However, the mathematical treatment applied by Zilversmit and co-workers (6) makes possible the calculation of the rate of formation of a substance from its immediate precursor. In reference to cortisol this makes feasible the calculation of the rates of formation of several of its metabolites such as THF, allo-THF and THE. Hence, one can add to the quantitative evaluation of urinary metabolites an additional rate parameter to aid in relating the metabolism of cortisol to the pathological condition of the individual. It cannot be emphasized too strongly, however, that such calculations attempt to describe the body pool as a *whole* and because of this gross treatment, changes in the kinetics of cortisol metabolism, which are small or slow in comparison to total changes, may be overlooked.

In the kinetic data derived for the two individuals receiving adrenocorticotropin (Table I), the criteria outlined by Zilversmit and associates (6) was met, and good agreement between pool size and urinary excretion data was obtained. This latter agreement is consistent with the concept that the miscible metabolite pools exist largely as glucuronides and that there is little or no discrimination by the kidney between the different metabolite-glucuronides.

The marked contrast between the type of data obtained from the *amounts of metabolite excreted per unit time* and from the *rates of formation* of metabolite is well illustrated for both patients in Table I. The relative proportion of THE : allo-THF *excreted* is 1.6 for M.L.P. and 0.91 for E.K., whereas the relative proportion of the *rates of formation* for THE : allo-THF is 4.0 for M.L.P. and 2.5 for E.K. It is obvious that an estimation of the relative rates of formation of metabolites based on their relative quantities in the urine can be grossly misleading.

Another interesting observation concerns the probable lack of chemical transformation of the steroid moiety once conjugation with glucuronic acid has taken place. The data of Figure 5, suggest that the steroid-glucuronic acid conjugate is not further altered at positions C-11 and C-20. In this figure it can be seen that upon the addition of unlabeled THF the specific activities of THE and cortolone are lowered. This is attributable to the formation of a small quantity of THE and cortolone from the sudden infusion of unconjugated, nonradioactive THF. Since the unconjugated THF is rapidly transformed into THF-glucuronide, the decreases in specific activity of THE and cortolone are abrupt. There is not a continuous fall in specific activity of THE and cortolone as one might expect if any substantial amount of conjugated THF of specific activity, 11 cpm per μg. (THF specific activity after administration of unlabeled THF) were *continuously* being transformed into THE and cortolone. After the administration of unlabeled THE similar results can be seen in the cortolone curve. Such observations are consistent with the concept that once conjugation with glucuronic acid has occurred no further alteration of the steroid metabolite occurs at the two positions, C-11 and C-20. In view of these considerations the *small* amount of conversion of one metabolite to another may be the result of the removal of metabolite by conjugation rather than the result of a lack of enzyme systems capable of performing the reversible reduction at position 11 or position 20.

The role of protein-binding in these studies is unknown. It would appear that if *in vivo* protein-binding of steroids is extensive (33, 34) its nature is such that it does not prevent either the ready mixing of cortisol and its metabolites or the rapid metabolism of cortisol.

Several investigators (1, 31, 35, 36) have suggested that the quantitative aspects of the metabolism of steroid hormones may aid in evaluating the role these hormones play in physiological processes. Hence, the results of these current studies are most encouraging. Further work correlating urinary excretion data, metabolite pool size, rates of formation of metabolites and metabolite interconversions in the steady state are in progress. It is necessary that extensive quantitative data, consistent in all aspects, be obtained to establish isotope dilution techniques for determining the rates of transformation of cortisol to specific metabolites in man.

SUMMARY

Several aspects of the kinetics of cortisol metabolism were investigated in normal adult volunteers after the rapid intravenous administration of trace amounts of cortisol-4-C^{14}. One group was maintained on a constant infusion of adrenocorticotropin begun four hours prior to the injection of cortisol-4-C^{14} and continued throughout the experiment.

The specific activities, counts per minute per microgram, of several cortisol metabolites were evaluated in urine samples collected every 15 minutes. Cortisol specific activities were determined in both plasma and urine. The metabolites evaluated were cortolone (3α, 11β, 17α, 20α or β, 21-pentahydroxy-5β-pregnane), tetrahydrocortisol (3α, 11β, 17α, 21-tetrahydroxy-5β-pregnan-20-one), allo-tetrahydrocortisol (3α, 11β, 17α, 21-tetrahydroxy-5α-pregnan-20-one) and tetrahydrocortisone (3α, 17α, 21-trihydroxy-5β-pregnan-11, 20-dione).

During the course of the experiments small amounts of nonlabeled metabolites were administered intravenously. From changes in specific activities of each metabolite and from the *amounts* of metabolite excreted per 15 minutes the following information was obtained:

Interconversion of metabolites. Two of the major metabolites of cortisol, tetrahydrocortisol and tetrahydrocortisone, are derived mainly from cortisol and cortisone, respectively. There is little interconversion of these two metabolites.

The specific activity of the metabolite, allo-tetrahydrocortisol, was not altered by the addition of either unlabeled tetrahydrocortisol, tetrahydrocortisone or β-cortolone. Hence, these metabolites are not converted to allo-tetrahydrocortisol The implications of this lack of conversion are discussed.

The fall in cortolone specific activity after the addition of unlabeled tetrahydrocortisol and tetrahydrocortisone suggests that these two compounds may be, to some extent, precursors of cortolone.

Pool sizes of metabolites. "Readily miscible" pool sizes of metabolites were calculated from the isotope dilution data and from urinary excretion values.

"Rates of formation" of metabolites. In two studies performed during adrenocorticotropin administration, the "rates of formation" (milligram per 24 hours) of tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone were computed from the kinetic data. The sum of the "rates of formation" of these three metabolites accounted for approximately 80 per cent of the total turnover of cortisol.

It is suggested that additional similar studies may aid in correlating cortisol metabolism with altered physiology in man.

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ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY: EVIDENCE OF DIFFERENCES BETWEEN NEGROES AND CAUCASIANS WITH RESPECT TO THIS GENETICALLY DETERMINED TRAIT *

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Erythrocyte glucose-6-phosphate dehydrogenase (G-6-P.D.) deficiency is associated with an increased susceptibility to hemolysis following ingestion of such agents as primaquine, naphthaline, sulfonamides, other drugs and the fava bean (1, 2). The incidence of this trait varies considerably among different population groups. Thus, it is relatively common among Negroes (3–7) and among Caucasians of Italian (4, 5), Greek (4, 5, 8) and Sephardic Jewish (9) extraction. On the other hand, the incidence of erythrocyte G-6-P.D. deficiency is low among subjects of Chinese (10) and Javanese (7) descent and is particularly rare among Ashkenazy Jews (9) and certain other Western Europeans (1–5). Data accumulated in both Negro (4, 5) and Caucasian populations (4, 5, 9) have suggested that this erythrocyte enzyme deficiency is due to a sex-linked gene of intermediate dominance.

It has been assumed that the deficiency in G-6-P.D. in these various population groups is a result of the same gene mutation. The explanation of the varied incidence of this enzyme defect in different racial groups has remained an intriguing problem.

The present investigation has been concerned with further characterization of the relationship between the gene alteration and the resulting deficiency in red blood cell G-6-P.D. Among subjects with low erythrocyte G-6-P.D. activity, Caucasian males were found to have a more marked deficiency of this enzyme than Negro males. Recently, it was reported (11) that Negro subjects with erythrocyte G-6-P.D. deficiency had normal levels of this enzyme in their leukocytes. It has now been found that affected [1] Caucasian males have significantly lower than normal activities of G-6-P.D. in their leukocytes.

Among affected subjects, the deficiency in G-6-P.D. activity is more marked in red blood cells than in leukocytes. In persons with normal erythrocyte G-6-P.D. activity, the level of this enzyme decreases with the *in vivo* aging of the red blood cells (12). In the present study the effect of age on erythrocyte G-6-P.D. activity has been examined in affected subjects. Enzyme activity was assayed in young and old red cell fractions separated by serial osmotic hemolysis or by centrifugation. G-6-P.D. activity in young red cells was found to be higher among affected Negro males than affected Caucasian males, and to decrease with the *in vivo* aging of the erythrocytes.

METHODS

Subjects studied. All subjects had normal values for hemoglobin concentrations and erythrocyte, reticulocyte, leukocyte and platelet counts with the exceptions of the patients with chronic hemolytic anemia (Table I) and those with reticulocytosis (Table IV). The subjects studied with erythrocyte G-6-P.D. deficiency are indicated in Footnotes † and ‡ of Table I. The control group included subjects selected at random from among hospital personnel and patients. Fifty-nine of the subjects included in Table I, and 31 of the control and 14 of the "deficient" Negro group included in Table II, represent persons previously reported (5, 11).

Enzyme assays. G-6-P.D. was assayed by a method based on that of Kornberg and Horecker (13). The details of this enzyme assay have been described else-

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[1] The term "affected" is used to denote subjects with erythrocyte glucose-6-phosphate dehydrogenase activities below 3.0 standard deviations of the control mean value.

where (14). A unit of G-6-P.D. activity in erythrocytes was defined as a change in one optical density unit per minute per gram of hemoglobin. Enzyme activity in erythrocytes was referred to hemoglobin concentration rather than cell count or cell volume because this parameter was found to provide most reproducible results (14, 15). A unit of G-6-P.D. activity in leukocytes was defined as the change in one optical density unit per minute per 10^6 cells. Prior to hemolysis of erythrocyte samples or disruption of white cell samples, aliquots of the suspended cells were removed for counting of erythrocytes, leukocytes and, when appropriate, reticulocytes and platelets. Hemoglobin concentrations were measured as previously described (12).

Preparation of cells. Venous blood, mixed with heparin to prevent clotting, was employed for all studies. Hemolysates of washed erythrocytes were prepared from the blood for enzyme assay as previously described (14). The washed erythrocytes were contaminated with fewer than 0.05 per cent leukocytes.

For studies in which enzyme activity was determined in both leukocytes and erythrocytes, venous blood was drawn into siliconized syringes and transferred to siliconized test tubes. The blood cells were separated by a method of dextran flotation (16) which was carried out at 4° C. The whole blood was added to an equal volume of 3 per cent dextran and 3 per cent glucose in 0.9 per cent sodium chloride and allowed to sediment for 30 to 45 minutes. The supernatant plasma, containing leukocytes and platelets with relatively few red cells, was removed and centrifuged at 800 × G for eight minutes. The sedimented white cells were recovered and suspended in approximately 20 volumes of isotonic potassium chloride buffered at pH 7.4 with potassium bicarbonate. The leukocytes were disrupted by freezing and thawing four times. Leukocyte counts were performed prior to and following this procedure to insure that assays were performed only on samples in which more than 90 per cent of the white cells were destroyed. This procedure yielded a leukocyte preparation contaminated with fewer than five red cells and less than one platelet for each white cell. This degree of contamination by erythrocytes and platelets had no detectable effect on the assays of leukocyte enzyme.

The erythrocytes, following sedimentation in dextran, were centrifuged at 1,000 × G for 10 minutes. The supernatant was removed and the red cells washed twice with isotonic potassium chloride buffered at pH 7.4 and resuspended in this solution. The erythrocyte samples prepared in this manner were contaminated with less than 0.03 per cent leukocytes and 0.5 per cent platelets. This degree of contamination by platelets had no detectable effect on the red cell enzyme assays. However, a detetcable level of G-6-P.D. was attributable to the leukocytes in these erythrocyte samples. The enzyme activity attributable to the leukocytes was calculated by multiplying the number of leukocytes in the erythrocyte sample by the enzyme activity per white cell. Among 82 subjects with a mean erythrocyte G-6-P.D. of 16.1 ± 2.4 units,[2] the enzyme activity attributable to leukocytes present in these red cell samples averaged 0.4 ± 0.2 unit. In 45 affected persons with an average red cell G-6-P.D. of 2.1 ± 1.6 units, G-6-P.D. attributable to leukocytes averaged 0.2 ± 0.15 unit. This method of correcting the erythrocyte enzyme activity for leukocyte contamination probably overestimated the contribution of the white cell enzyme. The erythrocytes were hemolyzed by freezing and thawing twice. This procedure usually disrupted only 60 to 75 per cent of the leukocytes. In addition, in seven Caucasian persons (included in Table I) there was no detectable red cell G-6-P.D. despite the fact that these samples were contaminated with 0.007 to 0.03 per cent leukocytes. These data indicate that the error in determining red cell G-6-P.D. activity attributable to white cell contamination was small.

Separation of young and old erythrocytes. The resuspended washed erythrocytes (to be referred to as the whole erythrocyte population) were separated into fractions of relatively young and old mean ages by two different methods: *a*) serial osmotic hemolysis and *b*) centrifugation.

a) Serial osmotic hemolysis: The method of graded osmotic hemolysis employed for the separation of the whole erythrocyte population into fractions of differing mean cell age has been previously described (17). This method is based on the fact that young mature human erythrocytes are more resistant to hemolysis in hypotonic media than old cells.

b) Centrifugation: A modification of previously published methods of separating the whole erythrocyte population into fractions of relatively young and old cells was employed (12, 18). This method is based on the fact that young red cells are less dense than old cells. Approximately 25 ml. of the whole erythrocyte population was centrifuged at 1,900 × G for 45 minutes in siliconized glass tubes of inner dimensions 7 × 145 mm. The upper 10 per cent and the lower 10 per cent of red cells, respectively, from each tube were combined and resuspended in isotonic potassium chloride buffered at pH 7.4. These samples were again centrifuged at 1,900 × G for 45 minutes, and the top 15 per cent of the upper red cell sample and the bottom 15 per cent of the lower red cell sample were recovered and washed once with potassium chloride buffered at pH 7.4 and resuspended in this solution. Aliquots of each sample were taken for cell counts, hemoglobin determination and enzyme assay.

RESULTS

Erythrocyte glucose-6-phosphate dehydrogenase activity

In a group of 500 adult subjects the erythrocyte G-6-P.D. activity was 16.1 ± 2.2 units. Among this control group no significant differences were found between the mean values for red cell G-6-

[2] The mean value ± on standard deviation.

P.D. of males compared with females, or Caucasians compared with Negroes. Subjects whose level of dehydrogenase in erythrocytes was less than 3.0 standard deviations below the control mean were considered to have a deficiency of G-6-P.D.

The activity of erythrocyte G-6-P.D. among affected Negro males was 2.7 ± 1.5 units compared with 0.4 ± 0.4 unit for deficient Caucasian males (Table I). The difference between these means was statistically highly significant, 11 times the standard error of the difference. The average red cell G-6-P.D. level for the group of affected Negro females was 6.9 ± 2.3 units compared to 6.8 ± 3.2 units for affected Caucasian females (Table I). The difference between these means was not statistically significant.

TABLE I

Erythrocyte glucose-6-phosphate dehydrogenase activity among subjects with a deficiency of this enzyme

Subject	No.	Erythrocyte glucose-6-phosphate dehydrogenase* Mean	Range	S. D.
Negro†				
Male	59	2.7	0.7–6.0	1.5
Female	52	6.9	2.0–9.5	2.3
Caucasian‡				
Male	22	0.4	0–1.1	0.4
Female	22	6.8	0–9.6	3.2

* Enzyme activity is expressed as the change in optical density units per minute per gram hemoglobin.

† Negro male subjects include: *a*) 24 persons without known hematological disorders who were discovered during a random population survey and 11 relatives of such subjects; *b*) 6 persons with a history of hemolytic anemia following ingestion of naphthaline and 8 relatives of such subjects; *c*) 1 person with a history of favism; *d*) 6 persons with a history of acute hemolytic anemia of undetermined cause and 3 relatives of such subjects. Negro female subjects include: *a*) 19 persons without known hematological disorders who were discovered during a random population survey and 14 relatives of such subjects; *b*) 1 person with a history of acute hemolytic anemia following ingestion of naphthaline and 10 relatives of such subjects; *c*) 2 relatives of subjects with a history of favism; *d*) 6 relatives of subjects with a history of acute hemolytic anemia of undetermined cause.

‡ Caucasian male subjects include: *a*) 4 persons with a history of favism and 7 relatives of such subjects; *b*) 3 persons with a history of hemolytic anemia following the ingestion of sulfonamides and 2 relatives of such a subject; *c*) 2 persons with a history of acute hemolytic anemia of undetermined cause; *d*) 2 persons with chronic hemolytic anemia of undetermined cause and 2 relatives of such subjects. Caucasian female subjects include: *a*) 2 persons without known hematological disorders who were discovered during a random population survey; *b*) 2 persons with a history of favism and 12 relatives of such subjects; *c*) 4 relatives of subjects with a history of acute hemolytic anemia of undetermined etiology; *d*) 2 relatives of subjects with chronic hemolytic anemia of undetermined etiology.

TABLE II

Leukocyte glucose-6-phosphate dehydrogenase activity among normal subjects and persons with a deficiency of this enzyme in their erythrocytes

Subject	No.	Leukocyte glucose-6-phosphate dehydrogenase* Mean	Range	S. D.
Control†	85	44.4	19.2–99.8	19.3
Negro	48	46.8	22.9–99.8	22.0
Caucasian	37	42.1	19.2–64.4	13.0
Deficient‡				
Negro	38	38.1	26.2–63.0	11.6
Caucasian	17	14.0	6.1–23.4	5.9

* Enzyme activity is expressed as change in optical density units per minute per 10^9 leukocytes.

† The mean ± standard deviation for erythrocyte glucose-6-phosphate dehydrogenase activity of the control Negro group was 16.2 ± 2.3 units and for the control Caucasian group, 16.1 ± 2.5 units.

‡ All subjects included in this group had erythrocyte glucose-6-phosphate dehydrogenase activity below 4.5 standard deviations of the control mean value.

Subjects with erythrocyte G-6-P.D. deficiency may be divided arbitrarily into two groups: a moderately deficient group, with values of red cell enzyme between 3 and 4.5 standard deviations below the control mean; and a severely deficient group, with enzyme activities lower than 4.5 standard deviations from the control means. The group of Negroes with a moderate deficiency in G-6-P.D. included 45 females and no males, while those with a severe deficiency of the red cell enzyme included 59 males and 7 females. In the group of affected Caucasians, those with a moderate deficiency included 19 females and no males and those with a severe deficiency included 22 males and 3 females. This preponderance of females among moderately affected subjects and of males among severely affected persons is in agreement with several previously published observations (4, 5, 9, 19). This observation is consistent with the suggestion that this trait is due to a sex-linked gene of intermediate dominance (4, 5, 9).

Leukocyte glucose-6-phosphate dehydrogenase

In Negro subjects with marked red cell G-6-P.D. deficiency, the activity of this enzyme in leukocytes was not significantly different from that in white cells of the control group (Table II). This finding is in agreement with an earlier report based on a smaller number of observations (11).

TABLE III

Glucose-6-phosphate dehydrogenase activity in erythrocyte fractions of different age separated on the basis of varying osmotic fragility: subjects with normal erythrocyte glucose-6-phosphate dehydrogenase

Negroes						Caucasians					
		Osmotic resistance†						Osmotic resistance			
		Most		Least				Most		Least	
Subject No.	WEP*	% Cells	G-6-P.D.	% Cells	G-6-P.D.	Subject No.	WEP	% Cells	G-6-P.D.	% Cells	G-6-P.D.
1	12.3	5	18.0	5	5.2	20	16.8	2	34.8	3	7.5
2	17.3	2	33.6	5	5.8	21	14.0	3	40.8	5	5.5
3	15.5	5	26.9	2	1.2	22	13.3	2	25.2	6	5.0
4	20.9	2	36.0	4	6.1	23	15.2	5	19.3	5	4.2
5	12.8	5	28.5	3	3.2	24	19.9	5	39.8	‡	‡
6	14.3	3	31.2	4	4.9	25	12.1	5	26.7	5	2.6
7	16.6	4	36.0	3	4.2	26	16.3	2	38.3	2	1.7
8	15.9	3	30.2	3	4.1	27	12.7	2	28.5	2	3.4
9	18.4	1	45.0	‡	‡	28	12.0	2	40.2	2	2.1
10	14.3	5	38.4	3	4.4	29	13.2	2	29 8	2	4 9
11	16.8	5	38.4	‡	‡	30	15.7	2	23.5	2	1.2
12	14.2	7	31.2	2	6.7	31	15.6	3	23.6	9	5.0
13	16.4	2	45.2	4	2.4	32	15.1	2	31.6	5	5.2
14	15.1	4	33.6	‡	‡	33	15.5	5	22.3	2	1.8
15	16.9	2	45.2	‡	‡	34	18.0	5	39.6	7	6.4
16	16.0	2	41.9	2	2.0	35	13.1	2	31.2	5	4.6
17	15.5	3	31.9	3	5.6	36	17.9	5	38.4	‡	‡
18	14.6	3	26.2	‡	‡	37	13.4	3	22.8	7	4.5
19	13.1	3	42.8	3	7.2	38	13.8	3	39.6	‡	‡
						39	17.2	4	30.0	‡	‡
						40	14.6	4	40.0	4	7.9
						41	14.2	3	39.6	5	8.9
						42	13.0	4	27.6	5	7.9
						43	17.8	2	32.4	3	7.4
						44	14.4	2	38.4	3	8.8
						45	12.2	5	21.4	5	3.4
						46	15.8	5	18.6	5	5.9
						47	13.7	3	40.2	2	6.1
						48	16.2	3	33.0	2	4.7
M	15.6	3	34.7	3	4.5	M	14.9	3	31.6	4	5.0
S. D.	2.1	2	7.2	1	1.7	S. D.	2.0	1	7.3	2	2.0

* Abbreviations: WEP, whole erythrocyte population; G-6-P.D., glucose-6-phosphate dehydrogenase. Enzyme activity is expressed as change in optical density units per minute per gram hemoglobin; M, mean value; S. D., standard deviation.

† In each fractionation the red cell sample obtained which was most resistant to hypotonic hemolysis (relatively young cells) and that which was least resistant to hypotonic hemolysis (relatively old cells) were assayed for glucose-6-phosphate dehydrogenase activity. The % cells refers to the percentage of the whole erythrocyte population in these fractions most and least osmotically fragile.

‡ No sample obtained for analysis.

It is to be noted, however, that the level of leukocyte dehydrogenase in these affected Negroes tended to be in the lower range of normal.

In contrast to these findings, Caucasians with a marked deficiency of erythrocyte enzyme had a mean value for white cell G-6-P.D. significantly lower than that of both the control Caucasian group and the deficient Negro subjects. The difference between the means for leukocyte G-6-P.D. activity of the affected Caucasians compared with the control group and with the affected Negro group was 10.9 and 10.2 times the standard error of the difference, respectively.

In seven Negro females whose erythrocyte G-6-P.D. activity was moderately decreased, the level of this enzyme in their leukocytes was within the range of values for the control group. No data were obtained for leukocyte G.-6.P.D. activities in Caucasians with a moderate decrease in erythrocyte enzyme.

Enzyme activity in erythrocytes fractionated by serial osmotic hemolysis

In red cells fractionated by serial osmotic hemolysis, the most resistant and least resistant cell fractions have relatively younger and older mean cell ages, respectively. Accordingly, the enzyme activities may be related to the relative mean age

TABLE IV

Glucose-6-phosphate dehydrogenase activity in erythrocyte fractions of different age separated on the basis of varying osmotic fragility: subjects with deficient erythrocyte glucose-6-phosphate dehydrogenase

Markedly G-6-P.D. deficient subjects*											
Negroes						Caucasians					
		Osmotic resistance						Osmotic resistance			
		Most		Least				Most		Least	
Subject No.	WEP†	% Cells	G-6-P.D.	% Cells	G-6-P.D.	Subject No.	WEP	% Cells	G-6-P.D.	% Cells	G-6-P.D.
1	1.8	6	4.9	1	0	12	0.7	3	1.0	3	0
2	4.6	5	12.5	5	0.5	13	0.1	6	1.5	3	0
3	1.9	4	7.7	8	2.6	14	0.5	5	1.9	5	0
4	6.0	1	17.0	4	4.4	15	0.5	8	1.0	5	0
5	2.4	4	19.4	2	1.0	16	1.6	8	1.2	8	0
6	1.8	7	6.0	8	1.2	17	0.2	5	0	6	0
7	1.3	5	1.5	3	0.6	18	0.3	3	1.1	9	0
8	1.8	5	4.2	3	0	19	0	2	0	2	0
9	1.6	4	1.7	2	0.3	20	2.2	5	1.9	8	0
10	1.2	3	2.6	2	0.5	21	0.4	2	1.6	10	0.2
11	2.4	9	16.8	7	1.4						
M	2.4	5	8.1	4	1.1	M	0.6	5	1.1	6.0	0
S. D.	1.5	2	6.3	2	1.2	S. D.	0.7	2	0.6	2	
Moderately G-6-P.D. deficient subjects											
22	9.0	7	32.5	5	3.7	33	9.1	2	28.8	6	7.6
23	9.3	2	20.0	3	5.3	34	8.3	5	20.0	6	6.8
24	9.3	3	20.0	6	4.0						
25	9.1	1	24.0	2	8.0						
26	7.3	3	18.7	3	4.8						
27	8.4	1	26.9	3	7.8						
28	9.0	2	21.6	4	5.2						
29	8.9	6	16.8	6	5.4						
30	8.4	10	14.5	2	6.5						
31	8.4	7	40.8	4	4.0						
32	8.8	7	13.5	5	6.8						
M	8.7	4	22.6	4	5.6						
S. D.	0.5	3	7.2	1	1.6						

* Markedly G-6-P.D. deficient subjects included persons whose erythrocyte enzyme activity was below 4.5 standard deviations of the control mean. Moderately G-6-P.D. deficient subjects included persons whose erythrocyte enzyme activity was 3.0 to 4 5 standard deviations below the control mean.

† See Footnotes * and †, Table III.

of the red blood cells in the various fractions. Among normal subjects the activity of G-6-P.D. was markedly higher in the younger than in the older erythrocyte fractions (Table III). The mean value for enzyme activity in the younger fraction exceeded that in the older cells by factors of 7.7 and 6.3 for the control group of Negroes and of Caucasians, respectively. There was variation among different subjects in the enzyme activity in erythrocyte fractions of the same osmotic resistance. This variation is attributable, in part, to the fact that such erythrocyte fractions prepared from red cell populations of different subjects vary in the degree of relative enrichment with young and old cells (17). However, in a given fractionation study, it was consistently observed that the older the relative mean cell age of the erythrocyte sample, the lower the activities of G-6-P.D. (12).

In Negroes with a marked erythrocyte G-6-P.D. deficiency, the average activity of this enzyme in younger cell fractions was significantly higher than in comparable samples prepared from severely affected Caucasians (Table IV). Among these affected Negroes, G-6-P.D. activities in the younger cell fractions did not approach values obtained for control subjects. Nevertheless, enzyme activity, on the average, was 7.4 times higher in the young compared with the old red cell fraction. Caucasians with very low G-6-P.D. activities in the whole erythrocyte population generally had little detectable enzyme activity in the younger cell fractions and relatively small differences between these erythrocyte samples and older cell fractions.

In Negroes with a moderate decrease in erythrocyte G-6-P.D. activity, the mean value for the enzyme in younger compared with the older erythrocyte fractions differed by a factor of 4.0 (Table IV).

Enzyme activity in erythrocytes fractionated by centrifugation

To examine further the effect of aging on enzyme activity in affected compared with control subjects, red blood cells from persons with a reticulocytosis were fractionated by centrifugation. This technique provides populations of cells enriched with young and old erythrocytes in an unlysed state permitting a determination of the reticulocyte count in the samples of erythrocytes assayed for enzyme activity.

In control subjects and persons with an increased percentage of reticulocytes, the level of G-6-P.D. was higher in the upper (younger) than in the lower (older) erythrocyte fractions (Table V). As previously observed (14), patients with reticulocytosis compared with control subjects, have higher levels of G-6-P.D. in their whole erythrocyte population as well as in the relatively younger and older red cell fractions. The fractions of erythrocytes of relatively young and older mean cell age separated by centrifugation showed smaller differences in enzyme activity than did the fractions of differing mean cell age prepared by serial osmotic hemolysis. This finding, too, is in agreement with earlier published data (12).

In two Negro subjects with marked erythrocyte

TABLE V

Glucose-6-phosphate dehydrogenase activity in erythrocyte fractions of different mean cell age separated on the basis of varying density

		WEP†		Top 3% cells‡		Bottom 3% cells	
Subject*	Race	Reticulocyte count	G-6-P.D.	Reticulocyte count	G-6-P.D.	Reticulocyte count	G-6-P.D.
Normals							
1	C	0.4	12.4	0.2	21.6	0.0	14.9
2	C	0.5	17.0	0.6	24.7	0.3	14.7
3	C	0.8	19.4	1.7	30.1	0.0	17.7
4	C	0.8	15.9	2.6	30.0	0.2	10.5
5	N	0.9	14.1	1.1	20.9	0.2	12.3
6	N	0.9	14.4	3.6	21.8	1.1	9.7
7	N	0.7	15.9	1.1	24.3	0.3	12.9
Mean		0.7	15.6	1.6	24.8	0.3	13.2
Reticulocytosis with high enzyme activity							
8	C	13.6	25.9	42.0	49.6	0.2	18.2
9	C	15.4	22.2	51.1	38.4	0.3	20.4
10	C	68.0	22.9	98.0	62.0	30.0	18.2
11	N	6.0	20.2	6.9	31.0	0.9	18.3
12	N	3.9	29.0	8.1	40.5	0.3	23.6
13	N	79.0	27.9	88.0	48.4	10.0	22.7
Mean		31.0	24.7	39.0	45.0	7.0	20.2
Reticulocytosis with low enzyme activity							
14	C	18.2	0.3	23.9	1.5	3.8	0
15	C	2.9	0.5	7.1	0.9	0	0
16	N	4.0	3.2	8.3	6.5	0.7	2.0
17	N	3.9	1.1	13.9	8.0	0.7	0

* Subjects with reticulocytosis included persons with chronic hemolytic anemia of undetermined etiology (Subjects 8, 9, 10, 13 and 14) and patients with anemia secondary to bleeding from a peptic ulcer (Subjects 11, 12, 16 and 17). Patient 15 had no other clinically apparent hematological abnormality.

† Abbreviations: see Footnote *, Table III.

‡ The top 3 per cent cells, erythrocyte fractions with younger mean cell age, and the bottom 3 per cent cells, erythrocyte fractions with older mean cell age, were prepared according to the method indicated in the text. The top 3% cells compared to the bottom 3% cells generally contained a larger number of leukocytes relative to the erythrocyte concentration. Accordingly, all erythrocyte enzyme activities presented in this table represent values which are corrected for the dehydrogenase activity attributable to the white blood cells present in the red blood cell samples. See text for the details of the calculation of this correction.

G-6-P.D. deficiency, younger red cell fractions were obtained with reticulocyte counts of 8.3 and 13.9 per cent, respectively. G-6-P.D. activities were distinctly higher in these younger compared with the older cells (Table V, Subjects 16 and 17). By comparison, in two severely affected Caucasian subjects, the younger cell fractions, with 23.9 and 7.1 per cent reticulocytes, respectively, had little G-6-P.D. activity.

DISCUSSION

Erythrocyte G-6-P.D. deficiency was first demonstrated by Carson, Flanagan, Ickes and Alving (20) in Negro subjects who had an increased susceptibility to hemolysis following primaquine administration. Subsequently, this trait (measured by reduced glutathione stability or G-6-P.D. activity in red cells) has been recognized among certain Caucasian, as well as other Negro populations (1–10). It is most likely that this enzyme deficiency is due to a sex-linked gene of intermediate dominance (4, 5, 9). The present study indicates that there is a significant quantitative difference between Caucasians and Negroes who have a deficiency in G-6-P.D. Thus, G-6-P.D. activity is markedly lower in the whole erythrocyte population, the young red cells and the leukocytes of affected Caucasian males compared with affected Negro males. A review of previously published data (5, 21–26) reveals that affected Caucasian males were generally reported to have markedly low, or undetectable levels of G-6-P.D., while affected Negro males were rarely observed with levels below 10 per cent of the control mean value.[3]

Affected Negro and Caucasian females were found not to differ significantly with respect to the degree of red cell enzyme deficiency. This may reflect the fact that affected females are predominantly heterozygous. In heterozygotes, the presence of a single normal gene could mask quantitative differences in the enzyme activity which might result from different mutant genes.

Numerous investigations, primarily with Neurospora and certain bacteria (27–32), have provided evidence which suggests that a given functional genetic unit controls the formation of only one enzyme. Such studies have also provided examples of organisms in which two or more different mutants have been found to be deficient in the same enzyme (28–31). On the basis of these observations, several genetic mechanisms could account for the quantitative difference between Caucasians and Negroes. Thus, these findings could represent different mutations occurring in the same region on the X chromosome concerned with the formation of G-6-P.D.

On the other hand, these differences might reflect the fact that more than one gene may affect the level of G-6-P.D. activity. It has been suggested that such genetic modifiers may be concerned with repair of an altered enzyme or with control of various quantitative aspects of enzyme formation or function (29). Conceivably, there is a second mutation in affected Negroes which results in partial restoration of enzyme activity.

Another possibility, based on findings such as those of Umbarger and Brown (33), is that two or more distinctly different proteins may be produced which catalyze the same reaction, in this instance, the oxidation of glucose-6-phosphate. The quantitative difference between affected Caucasians and Negroes might then represent mutations involving different genes controlling the formation of the different proteins. Such mutations might result in varying degrees of decrease in the amount of enzyme activity.

The effects of a mutation which lead to a deficiency in enzyme activity appear to fall into at least two categories: *a*) partial or complete failure to produce the enzyme (27–32), or *b*) formation of a qualitatively altered protein (34–37). A partial or complete block in enzyme synthesis could result from either one of the genetic mechanisms mentioned above. However, in affected Negroes, the finding of normal, or near normal, G-6-P.D. activities in leukocytes suggests that, at least in this group, a block in the formation of this enzyme may not be the cause of the decreased levels of red cell enzyme.

Among affected subjects, G-6-P.D. deficiency was more marked in erythrocytes than in white cells. It has been suggested that a gene action leading to a formation of an altered enzyme of reduced stability would be compatible with these findings (11). In red cells, which synthesize little

[3] Ramot, Szeinberg and Sheba have observed that among Israeli subjects with erythrocyte G-6-P.D. deficiency, the level of this enzyme in leukocytes was significantly below that of a control group (personal communication).

or no protein, a faster than normal rate of decline in enzyme activity with aging *in vivo* could lead to a more marked deficiency of this enzyme in erythrocytes than in tissues which can synthesize proteins.

The evidence obtained in this study is inconclusive as to whether or not, in affected subjects, G-6-P.D. activity decreases more rapidly than normal with red cell aging. In normal and severely affected Negroes, G-6-P.D. activity in young compared with old erythrocyte fractions differed by essentially the same factor. However, in many of these affected subjects and in all of the severely affected Caucasians studied, the enzyme activity in the young cell fractions was very low. Accordingly, it is difficult to evaluate the meaning of the relatively small differences in enzyme activity between erythrocyte fractions enriched with young or old cells. These results could be compatible with erythrocyte G-6-P.D. deficiency being secondary to either *a*) a block in enzyme synthesis without any necessary alteration in its rate of degradation, or *b*) a more rapid than normal decrease in enzyme activity resulting in low levels in the very young red cell. In the latter case, G-6-P.D. activity in young cell fractions being very low, the relative differences between young and old cells might even be less than normal.

Studies by Kirkman (38) and in this laboratory (39) have, to date, revealed no difference in the properties of the G-6-P.D. purified from red cells of normal and of affected Negroes. Thus, the enzymes were found to have identical affinities for their substrates, glucose-6-phosphate and triphosphopyridine nucleotide, various triphosphopyridine nucleotide analogs, and pH optima. However, erythrocyte G-6-P.D. of affected compared with that of normal subjects has a greater heat-lability upon incubation of crude hemolysates at 37°C. (21, 40–42). This heat-lability of the enzyme from affected subjects is markedly decreased by addition of triphosphopyridine nucleotide (42). No evidence has been obtained to indicate that the difference in heat-lability between the normal and mutant enzymes reflects the presence of an inhibitor or lack of an activator in red cells of affected subjects (2, 39). It is possible that in affected Negroes (data on the enzyme purified from affected Caucasians are lacking) an altered enzyme is formed which, although unchanged in the specificity of its catalytic site, has a decreased stability at 37° C.

The present data permit no conclusions as to which, if any, of the above indicated genetic mechanisms accounts for the quantitative difference between affected Negroes and Caucasians. Further, the affected Negro and the affected Caucasian group may in themselves be genetically heterogeneous. However, it seems likely that elucidation of the mechanism of erythrocyte G-6-P.D. deficiency affords a model for extending our understanding of the nature of gene action in human subjects.

It is pertinent to point out that this study provides no information as to the relationship between erythrocyte G-6-P.D. deficiency and sensitivity to hemolysis. As summarized in recent reviews (1, 2, 19), the elucidation of certain biochemical abnormalities in red cells of drug sensitive subjects has not established the mechanism of their increased susceptibility to hemolysis. Accordingly, it is not possible to answer the question of whether the quantitative differences between affected Caucasians and Negroes is correlated with variations in the clinical course of the hemolytic reaction.

SUMMARY

Among subjects with a genetically determined deficiency in glucose-6-phosphate dehydrogenase (G-6-P.D.), there is a quantitative difference between Caucasians and Negroes. Affected Caucasian males compared with affected Negro males were found to have a significantly lower G-6-P.D. activity in the whole erythrocyte population, the young red cells, and the leukocytes. In affected Negro subjects, G-6-P.D. activities in young red cells, though not approaching control levels, were distinctly higher than in old cells. Caucasian males, whose whole erythrocyte population had little or no detectable G-6-P.D. activity, showed relatively small differences in the amount of enzyme activity detectable in young compared with old cells. It is suggested that different genetic mechanisms account for the deficiency of G-6-P.D. in Negroes and in Caucasians.

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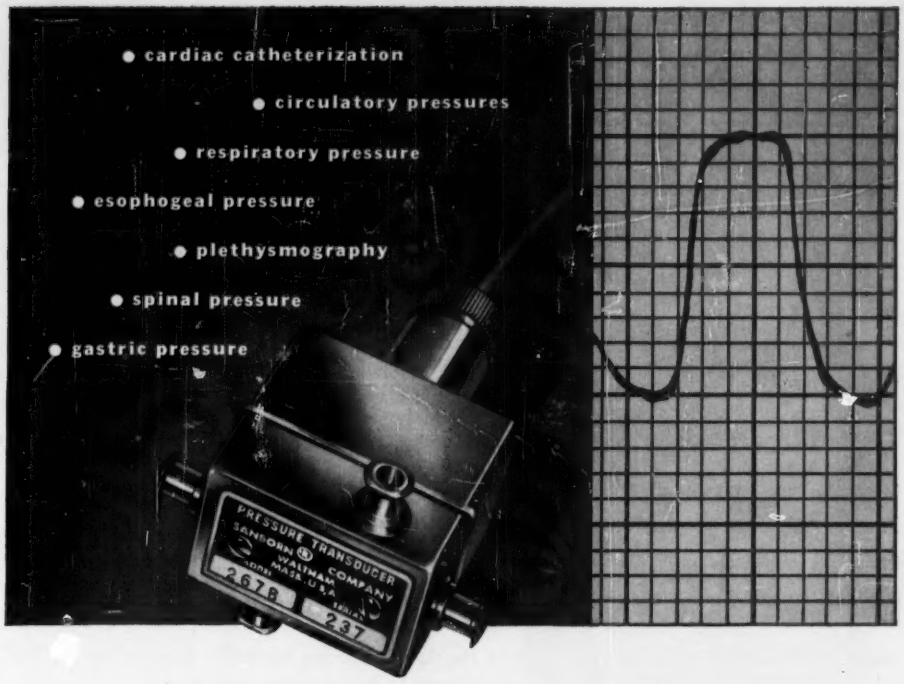

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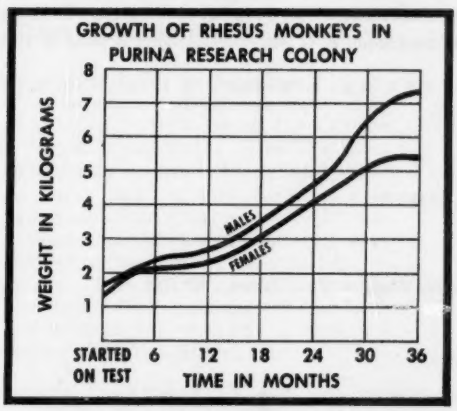

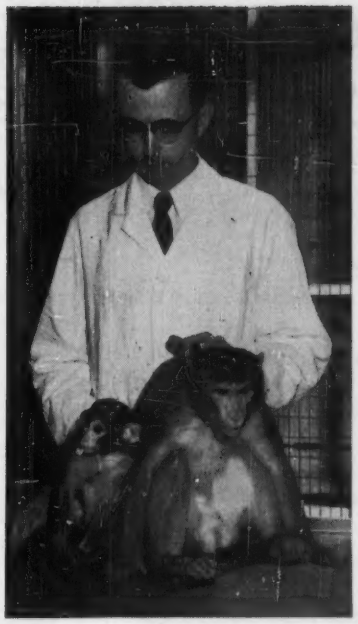

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Original Articles

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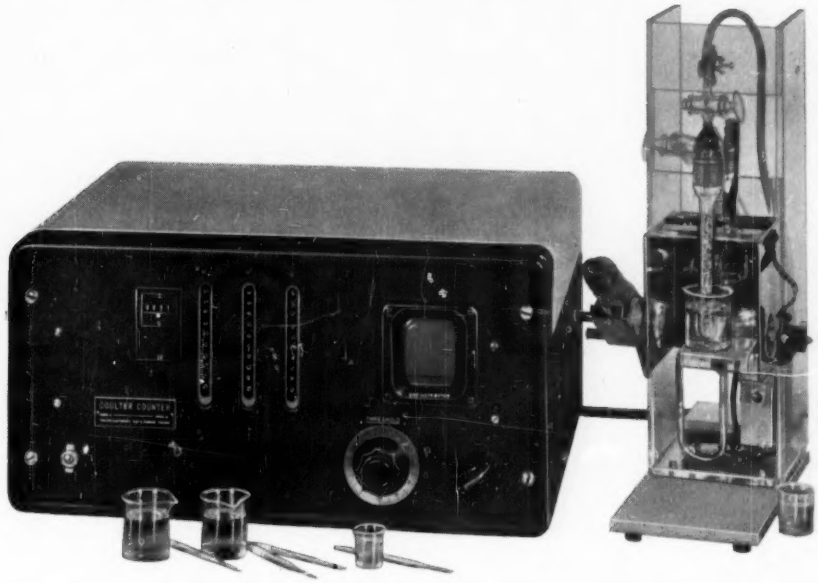

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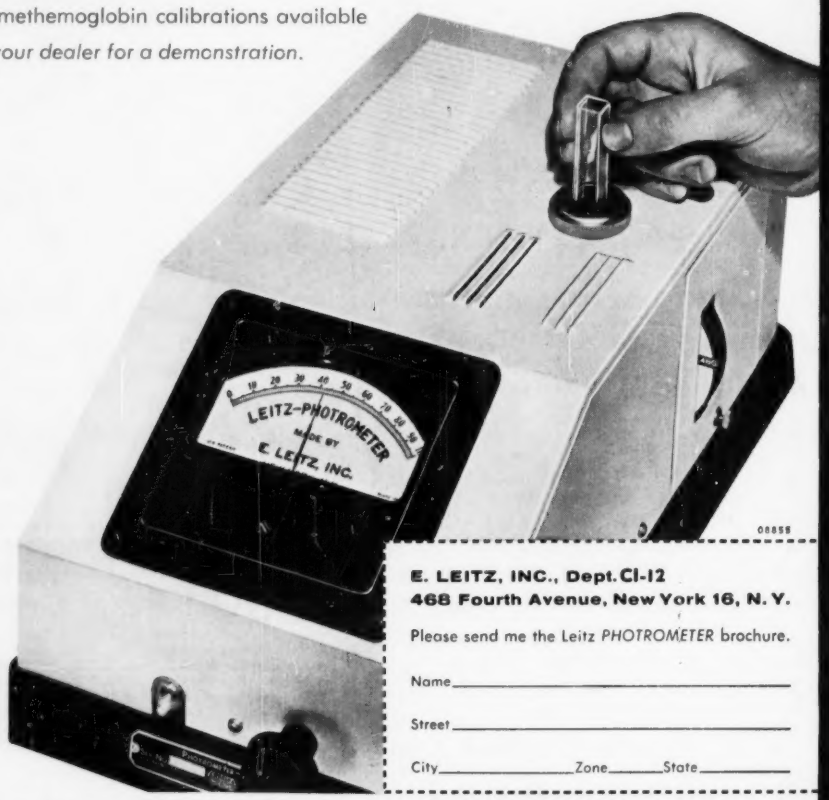

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